THE REGULATION OF NEUTROPHIL PRIMING AND ACTIVATION

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DECLARATION

This thesis was composed entirely by myself on the basis of work carried out under the supervision of Dr. Edwin R. Chilvers and Professor Christopher Haslett in the Rayne Laboratory, Respiratory Medicine Unit, University of Edinburgh.

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ABSTRACT

Neutrophils are a crucial component of the innate immune response and are responsive to a wide range of pro-inflammatory signals. However, the response of a neutrophil to a secretagogue agonist can be "primed" by pre-exposure to certain agents, including platelet-activating factor (PAF), tumour necrosis factor-α (TNFα) and lipopolysaccharide (LPS). Although priming refers specifically to the enhanced responses observed when neutrophils are subsequently stimulated, other functional responses are also associated with the primed state. These include cell polarization and increased neutrophil adhesiveness, which promote their recruitment to sites of inflammation. However, despite the undoubted beneficial and protective effects of neutrophils, these cells are paradoxically involved in the pathogenesis of a variety of inflammatory conditions. Furthermore, neutrophil priming has been shown to be an important prerequisite for neutrophil-mediated host tissue damage. Since primed neutrophils have been identified in the blood of patients with ARDS and inflammatory bowel disease, and in the joints of people with active rheumatoid arthritis, this functional upregulation of neutrophils is believed to contribute to the pathology of these disease states.

The potential for neutrophils to recover from a primed state, i.e. to "de-prime", remains largely unexplored. However, the priming of neutrophils under non-physiological conditions, using hypotonic buffers, has been shown to reverse upon the restoration of isotonicity. Having confirmed this finding, I demonstrated that the priming of human neutrophils with PAF, a well-established, rapid-acting, receptor-mediated priming agent, was transient with complete reversal to an un-primed state within 2 hours. Furthermore, de-primed neutrophils retained their capacity to be re-primed when subsequently challenged with either PAF or TNFα. The recovery of neutrophils to an un-primed state was confirmed by the assessment of superoxide anion release, cell polarization, and CD11b/CD18 function, and was shown to reflect neither a reduction in neutrophil viability or cell responsiveness, nor the metabolism

of PAF. These observations implied that neutrophil priming to a receptor-mediated agent was fully reversible. Transient neutrophil priming was also observed with inositol hexakisphosphate ($InsP_6$), a ubiquitous inositol polyphosphate whose minimal priming effects were found to occur independently of specific $InsP_6$ receptors on the surface of human neutrophils. In contrast, the receptor-mediated priming effects of TNFα were both slower to evolve and more sustained than those of either PAF or $InsP_6$. However, the primed responses elicited by PAF or TNFα could be manipulated by specific receptor blockade.

It has been suggested that a prolonged state of neutrophil priming might be an important component of the long-term inflammatory response observed with agents such as endotoxin (LPS). Thus, the recognition of a novel process whereby neutrophils have the potential to de-prime may represent a physiological and potentially targetable mechanism, upstream of the final activation pathway, to counteract the pro-inflammatory activity of neutrophils *in vivo*.

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TABLE OF CONTENTS

ABBREVIATIONS

ACLB	Albumin-coated latex beads
AEBSF	4 (2-aminoethyl) benzenesulfonyl fluoride
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C-PAF	1-*O*-alkyl-2-*N*-methylcarbamyl-glycerophosphocholine
C5a	Complement fragment 5a (anaphylotoxin)
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Intracellular free calcium concentration
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol (B-aminoethylether)-*N*,*N*-tetraacetic acid
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
G-protein	Guanine nucleotide binding protein
GDP	Guanine diphosphate
GTP	Guanine triphosphate
G-CSF	Granulocyte-colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Human albumin
HBSI	HEPES (20 mM)-buffered saline containing 50 μg/ml leupeptin, 20 μg/ml aproptinin, 1 mM AEBSF)
HBSS	Hanks' balanced salt solution
HEPES	*N*-2-hydroxyethylpiperazine-*N*-ethane sulphonic acid
ICAM-1	Intercellular cell adhesion molecule-1

IFNγ	Interferon-γ
$Ins(1,4,5)P_3$	Inositol 1,4,5-trisphosphate
$Ins(1,3,4,5,6)P_5$	Inositol pentakisphosphate
$InsP_6$	Inositol hexakisphosphate
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
LAD	Leukocyte adhesion deficiency
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LSB	Laemmli sample buffer (2×, 0.125 M TRIS-HCl, 4% SDS, 20% glycerol, 2.5 mM dithiothreitol, 0.01% bromophenol blue, 50 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM AEBSF, pH 6.8)
LTB_4	Leukotriene B_4
mAb	Monoclonal antibody
MOPS	3-[*N*-morpholino]propane sulphonic acid
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NKET	20 mM NaCl, 100 mM KCl, 5 mM EDTA, 20 mM TRIS, pH 7.7
O_2^-	Superoxide anion
PA	Phosphatidic acid
PAF	Platelet-activating factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3-hydroxykinase
PLA_2	Phospholipase A_2

PLC	Phosphoinositide-specific phospholipase C
PLD	Phospholipase D
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TNFα	Tumour necrosis factor-α

1. CHAPTER 1: INTRODUCTION

1.1 The Neutrophil

Neutrophils are leukocytes that are the first cells to be recruited to a site of inflammation (Schleimer *et al.,* 1989): hence, they constitute a fundamental component of the innate immune response. Their primary function is to phagocytose and degrade cell debris and any material which is foreign to the host (e.g. bacteria). The functional capacity of neutrophils depends upon several main processes: (i) chemotaxis towards the inflammatory site; (ii) phagocytosis of the offending agent; (iii) degranulation, with the release of pre-formed enzymes and proteins from intracellular granules; (iv) the respiratory burst, with the generation of highly microbicidal reactive oxygen species (ROS); and (v) the *de novo* generation of inflammatory mediators.

The important role of the neutrophil in acute inflammation has been inferred from the recurrent infections, especially with pyogenic bacteria, encountered by patients with abnormal neutrophil function. This includes patients with reduced numbers of circulating neutrophils (neutropaenia) (Bodey *et al.,* 1966), as well as those with isolated defects of neutrophil function, for example of adherence (e.g. leukocyte adhesion deficiency, LAD (Anderson and Springer, 1987)), migration and chemotaxis (e.g. Chediak-Higashi syndrome (Rausch *et al.,* 1978)), microbicidal activity (e.g. chronic granulomatous disease (Thrasher *et al.,* 1994)) and phagocytosis (e.g. diabetes mellitus). However, whilst neutrophils are essential for host defence, their inappropriate or excessive activation may, paradoxically, also contribute to the pathology of various inflammatory conditions (see 1.4). Indeed, the regulation of neutrophil activation has been described as a "double-edged sword" (Smith, 1994), defining a fine balance between the defence and damage of host tissues.

This Chapter will outline the primary role of the neutrophil as a professional phagocyte. It will commence with the neutrophil's origin in the bone marrow and subsequent release into the circulation; thereafter, it will consider the neutrophil in the context of an acute inflammatory response, with its recruitment from the circulation and priming/activation in the tissues, to its inevitable death. Although acute inflammation also comprises various vascular events, including vasodilatation and increased vascular permeability which increase the delivery of leukocytes and mediators to the inflammatory site, these will be discussed only where relevant to neutrophil function.

1.2 The Generation of Neutrophils

Neutrophils are produced from pluripotent stem cells within the bone marrow, under the influence of granulocyte- and granulocyte-macrophage colony-stimulating factors (G-CSF and GM-CSF, respectively). Mature neutrophils are released into the circulation as terminally-differentiated cells, at a rate of 10^{11} cells per day (Cannistra and Griffin, 1988), where they represent 50-60% of the total circulating leukocyte pool. They have a lobular nucleus identifying them as polymorphonuclear leukocytes, and are packed with cytosolic granules (Table 1.1) whose contents are essential for microbicidal killing. However, the circulating neutrophil count reflects a dynamic balance between three separate neutrophil pools residing in: (i) the bone marrow; (ii) the vasculature (which includes a circulating and a marginated pool); and (iii) the tissues. Indeed, neutrophils spend a relatively brief time within the circulation, with an estimated vascular half-life of 6-7 hours (Mauer *et al.*, 1960; Athens *et al.*, 1961).

Table 1.1 The Classification of Neutrophil Granules

(Borregaard *et al.*, 1993)

Granule Type	Main Contents	Main Functions of Contents
• **Secretory vesicles**	CD11b/CD18 (Mac-1)	Endothelial Adhesion
	fMLP-receptors	Chemoattraction
	FcγRIII	Antibody recognition
	Cytochrome b_{558}	Respiratory burst activity
• **Azurophilic (primary)**	Myeloperoxidase	Microbicidal
	Lysozyme	Microbicidal
	Defensins	Microbicidal
	Cationic proteins	Microbicidal
	Elastase	Proteolytic
	Cathepsins	Proteolytic/Hydrolytic
	β-glucuronidase	Hydrolytic
• **Specific (secondary)**	Lactoferrin	Microbicidal
	Lysozyme	Microbicidal
	CD11b/CD18	Endothelial Adhesion
	Collagenases	Extravasation
	fMLP-receptors	Chemoattraction
	Cytochrome b_{558}	Respiratory Burst Activity
• **Gelatinase (tertiary)**	Gelatinase	Extravasation
	CD11b/CD18	Endothelial Adhesion
	fMLP-receptors	Chemoattraction

1.3 The Role of the Neutrophil in Host Defence

The delivery of leukocytes to sites of inflammation (*extravasation*) is a critical function of the acute inflammatory response. Neutrophil extravasation comprises a sequence of events (Figure 1.1): (i) *margination* and *rolling* along the vascular endothelium; (ii) *firm adhesion* to the endothelial surface; and (iii) *trans-migration* across the endothelium.

1.3.1 Neutrophil Margination and Rolling

Under normal, laminar flow, leukocytes and erythrocytes travel in an axial stream within the vasculature, leaving a boundary layer of plasma in contact with the endothelium. However, in the early stages of an inflammatory response, leukocytes fall out of the axial stream and marginate towards the endothelial surface. This margination is particularly important in the microvascular beds of the lung, spleen and liver, where neutrophils are temporarily sequestered. The transient adherence of neutrophils to the activated endothelium results in their rolling along the walls of the microvasculature, especially the postcapillary venules (and small pulmonary capillaries) (Schmid-Schonbein *et al.,* 1980; Atherton and Born, 1973). Neutrophil rolling is thought to involve low-affinity, transient interactions of L-selectin (CD62-L), expressed on the tips of neutrophil microvilli (Borregaard *et al.,* 1994; Picker *et al.,* 1991), with sulphated glycoproteins on the endothelial surface (Rosen, 1994). There may also be contributions from P- and E-selectin, which are up-regulated on the surface of activated endothelial cells, and interact with oligosaccharides (such as Lewis antigen (Le^x) and sialyl-Le^x) on the neutrophil (Abbassi *et al.,* 1993; Lawrence and Springer, 1993; Bevilacqua and Nelson, 1993).

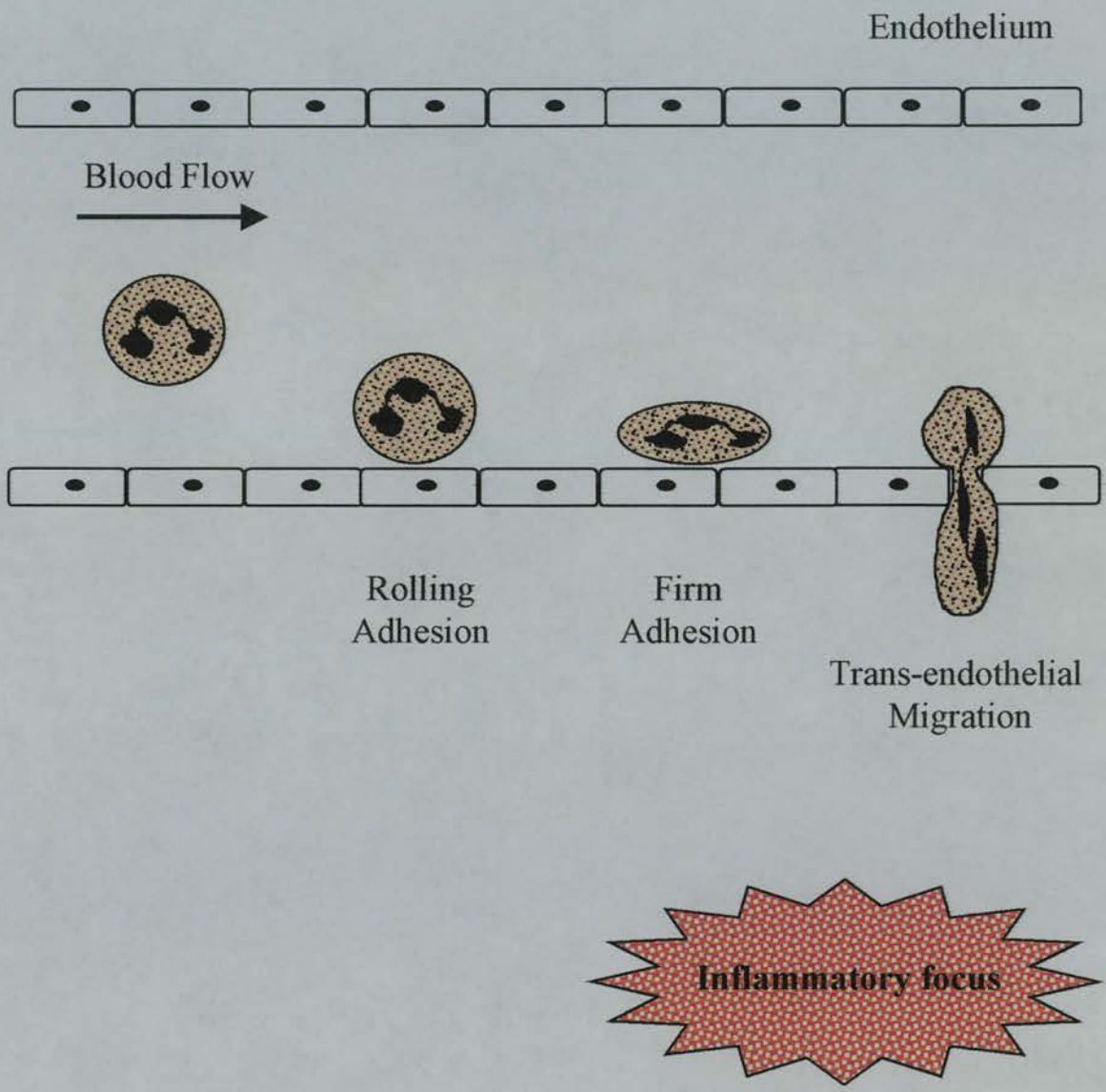


Figure 1.1
Schematic Representation of Neutrophil Extravasation.
Neutrophil recruitment to an inflamed site requires a series of adhesion and morphological events mediated by both the neutrophil and the vascular endothelium.

1.3.2 Firm Adhesion to the Endothelium

The low-affinity adhesive events that initiate neutrophil rolling can slow, but not stop, marginated neutrophils (McEver, 1993; Lasky, 1992). The complete arrest of neutrophils requires their firm adhesion to the endothelium, and is controlled by mediators generated during an acute inflammatory response. Chemotactic factors signal the recruitment of neutrophils to the inflammatory site, and may originate from various sources, including: (i) infectious agents at the inflammatory site (e.g. fMLP, LPS); (ii) previously activated leukocytes and endothelial cells (e.g. TNFα, GM-CSF, IL-8, PAF, LTB_4); and (iii) activated plasma components (e.g. C5a). Although the majority of these agents diffuse from their source to act as soluble mediators, some may be sequestered (in solid phase) on the surface of activated cells. For example, newly-synthesized PAF is co-expressed with P-selectin on the surface of activated endothelial cells, where it may trap and stimulate rolling neutrophils (Lorant *et al.,* 1993; Lorant *et al.,* 1991): this is an unusual example of *juxtacrine* stimulation (Zimmerman *et al.,* 1993) where a signalling molecule remains associated with the surface of the signalling cell (Figure 1.2).

Upon stimulation, the rapid exocytosis of intracellular granules, especially secretory vesicles (Borregaard *et al.,* 1994; Sengelov *et al.,* 1993), results in the fusion of granule membrane with the neutrophil plasma membrane. This increases the surface expression of β_2-integrins (especially CD11b/CD18) which are required for the firm adhesion of neutrophils to the endothelial surface (Smith, 1990; Anderson *et al.,* 1985). However, the increased surface expression of CD11b/CD18 alone does not ensure neutrophil adherence to the endothelium (Hughes *et al.,* 1992; Vedder and Harlan, 1988). Indeed, CD11b/CD18 must be modified at the plasma membrane before it becomes functional: this may be a result of phosphorylation (Buyon *et al.,* 1997), or increased expression of integrin-modulating factor-1 (Hermanowski-Vosatka *et al.,* 1992). The up-regulation of CD11b/CD18 avidity and expression on stimulated neutrophils is accompanied by an increased expression of the complementary intracellular adhesion molecule-1 (ICAM-1) on the surface of

activated endothelial cells (Springer, 1990). The prolonged, high-affinity nature of these neutrophil-endothelial interactions mediates the firm adhesion of neutrophils to the endothelium (Calafat *et al.,* 1993; Tonnesen, 1989). Neutrophil priming/activation also results in the shedding of L-selectin from the neutrophil surface (Stocks *et al.,* 1995; Lasky, 1992) which is a further prerequisite for trans-endothelial migration of these cells (Kuhns *et al.,* 1995).

The progression of the inflammatory response leads to the increased synthesis and expression of additional endothelial adhesion molecules, including E-selectin, that promote neutrophil rolling, thereby facilitating further adhesion to the activated endothelium (Bevilacqua and Nelson, 1993). Neutrophils may also adhere to the endothelium via bridging molecules, such as fibronectin, fibrin, and complement fragments (Marks *et al.,* 1991).

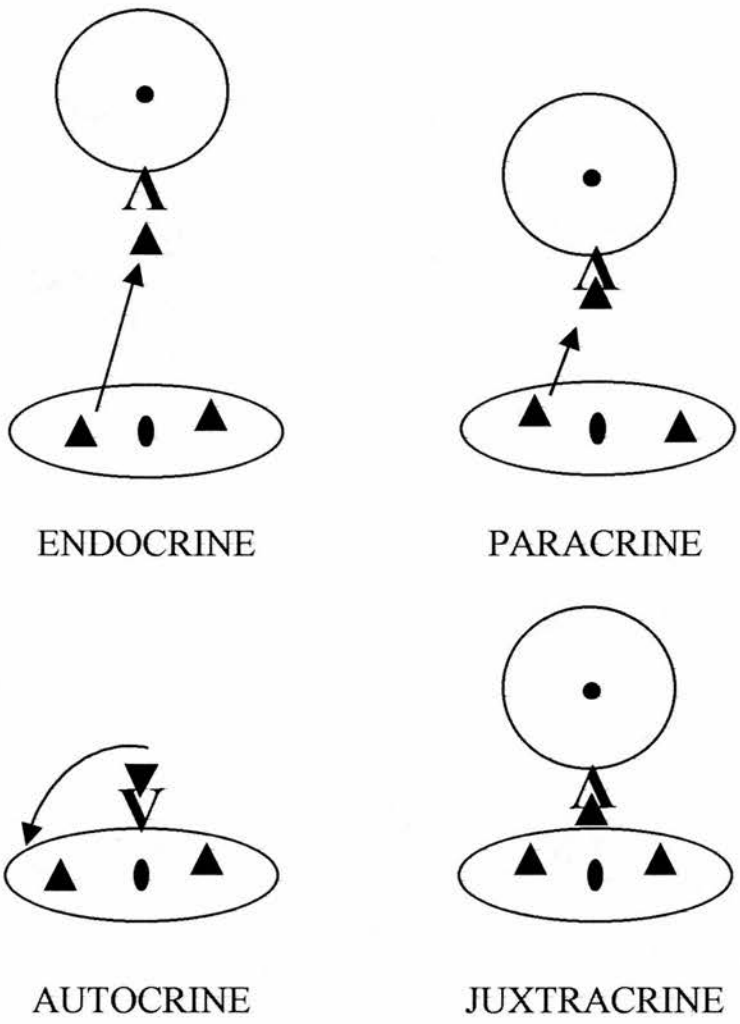


Figure 1.2
Mechanisms of Cellular Signalling by Bioactive Mediators.
In the juxtacrine mechanism, the signalling molecule remains associated with the surface of the signalling cell, rather than being released to activate the target cell in the fluid phase. This distinguishes juxtacrine interactions from endocrine, paracrine, and autocrine signalling mechanisms. (After Zimmerman et al., 1993).

1.3.3 Neutrophil Shape Change

Quiescent neutrophils are spherical cells. Approximately 20-30% of their total actin content is held as polymerized F-actin and contributes to the cytoskeletal network that lies adjacent to the plasma membrane (Sheterline *et al.,* 1984). Since this intracellular cytoskeleton interacts with the plasma membrane through integral membrane proteins, including integrins (Juliano and Haskill, 1993) and fMLP receptors (Jesaitis and Allen, 1988), it has been proposed to stabilize the membrane against external forces and have a role in the transduction of extracellular signals to the cell interior (Hynes, 1992). However upon neutrophil stimulation, the incorporation of secretory vesicle membrane into the neutrophil plasma membrane (Sengelov *et al.,* 1993) results in membrane ruffling (Hoffstein *et al.,* 1982). Furthermore, the rapid recruitment of monomeric G-actin from the cytosol into cytoskeletal F-actin produces focal alterations in the sub-membranous cytoskeleton, which are a prerequisite for the ensuing neutrophil stiffening, shape change and motility responses (Pecsvarady *et al.,* 1992; Worthen *et al.,* 1989). These morphological changes (particularly the reduced neutrophil deformability) facilitate the margination of neutrophils in the microvasculature, particularly in the small vessels of the lung (Schmid-Schonbein *et al.,* 1980).

1.3.4 Trans-Endothelial Migration

In order to leave the vascular space, neutrophils must squeeze through the inter-endothelial junctions of systemic post-capillary venules or pulmonary capillaries. Thus, they adopt a polarized morphology, with an elongated cell body, a broad, anterior lamellipodium and a small, uropod tail. Trans-endothelial migration is dependent upon directional cues from chemotactic factors and involves little random migration (chemokinesis) (Kitayama *et al.,* 1997). Although this process has not been fully characterized, it is known to require high-affinity interactions between neutrophil β_2-integrins and endothelial ICAM-1, and between glycosylated aminoglycans on the neutrophil plasma membrane and PECAM-1 (platelet

endothelial cell adhesion molecule), which is located in the inter-endothelial junctions (Muller *et al.,* 1993). After traversing the endothelial junctions, neutrophils are retarded transiently by the basement membrane (which forms a contiguous barrier beneath the vascular endothelium), but eventually pierce it, possibly aided by the secretion of granule contents such as gelatinase (Weiss *et al.,* 1986). Furthermore, it has been suggested that the endothelium, itself, may play a major role in the local degradation and reformation of the vascular basement membrane (Huber and Weiss, 1989).

1.3.5 Neutrophil Chemotaxis

In the tissues, neutrophils are guided towards an inflammatory focus by a gradient of chemotactic factors. The accuracy of this orientation correlates with the number of occupied receptors across the neutrophil surface (Zigmond *et al.,* 1981). The binding of chemotactic factors continues to promote the recruitment of secretory vesicles and specific granules to the neutrophil plasma membrane, with the consequent up-regulation of surface receptors, e.g. for fMLP (Fletcher and Gallin, 1983; Jesaitis *et al.,* 1982). This increased receptor expression occurs primarily at the leading edge of the lamellipodium (Nunoi *et al.,* 1985), whilst down-regulated receptors may be cycled to the trailing uropod and shed (Devreotes and Zigmond, 1988). These events are accompanied by dynamic morphological alterations in the lamellipodium, mediated by cycles of actin-dependent protrusion and contraction (Cassimeris *et al.,* 1990; Zigmond, 1993). Furthermore, neutrophil integrins mediate transient interactions with extracellular matrix proteins (Fuortes *et al.,* 1993; Hendey *et al.,* 1992), providing the traction required for locomotion.

1.3.6 The Phagocytosis of Opsonized Particles

The neutrophil is a professional phagocyte, and therefore has a primary function to recognize, phagocytose, and destroy any agent which is foreign to the host. The continued exocytosis of secretory vesicles and specifc granules to the neutrophil

surface, results in the up-regulation of CR1 and CR3 (CD11b/CD18), and FcγRIII (Tosi and Zakem, 1992): these are receptors for the complement components C3b and C3bi, and Fc fragments of immunoglobulin G (IgG), respectively, which act as opsonins by binding to foreign particles and marking them for phagocytosis. Thus, once the neutrophil has reached the inflammatory site it can identify particles that have been opsonized or certain lectins on the surface of micro-organisms (via non-specific glycosylated receptors). This recognition process triggers the extrusion of pseudopodia and engulfment of the target into a phagocytic vesicle (Figure 1.3).

1.3.7 The Respiratory Burst and Degranulation

The process of phagocytosis, together with the high, local concentrations of soluble stimuli at the inflammatory focus, initiates the transmembrane assembly of the NADPH oxidase enzyme system from its component parts (Babior, 1994). These parts include resident plasma-membrane components (the cytochrome b_{558} subunits $p91^{phox}$ and $p21^{phox}$) (phox = *ph*agocytic *ox*idase) and cytosolic factors ($p47^{phox}$, $p67^{phox}$ and $p21^{rac}$) (Rotrosen *et al.,* 1993). The functional NADPH oxidase then catalyzes an electron transport chain, using NADPH (generated by the cytosolic, hexose monophosphate shunt) as an electron donor (Babior *et al.,* 1973), to reduce oxygen to superoxide anions (O_2^-):

$$NADPH + H^+ + 2O_2 \rightarrow NADP^+ + 2H^+ + 2O_2^-$$

This correlates with an intense consumption of oxygen by the cell, which is referred to as the "respiratory burst" (Baldridge and Gerard, 1933). Superoxide anions are pumped into the phagocytic vesicle, where they dismute in the presence of the enzyme superoxide dismutase, producing hydrogen peroxide (H_2O_2):

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

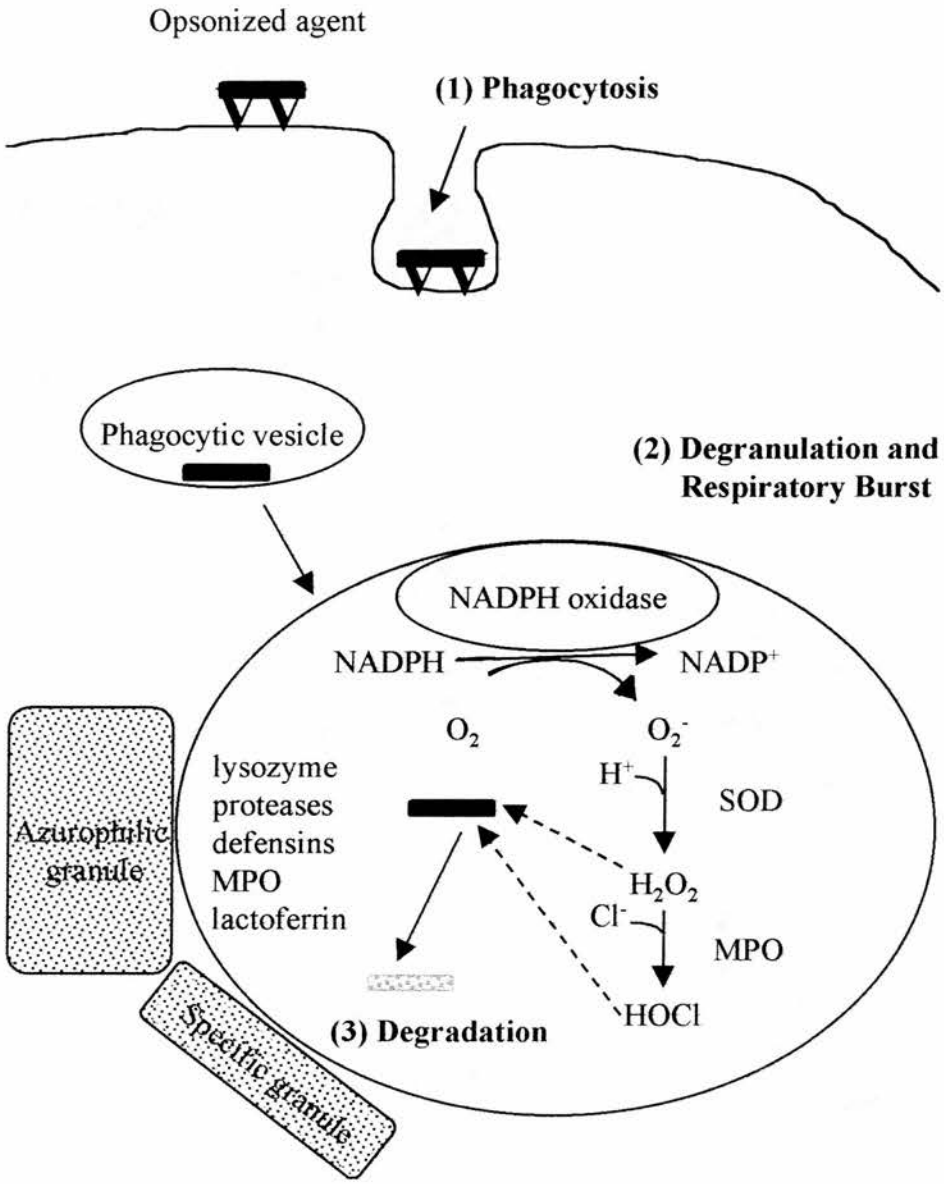


Figure 1.3
Schematic Representation of Phagocytosis and Microbial Killing.
(1) The recognition of opsonized agents by neutrophils at the inflammatory site initiates the process of phagocytosis. (2) The release of granule contents into the phagocytic vesicle together with the activation of NADPH oxidase generates a highly microbicidal environment (phagolysosome). (3) This results in the killing and degradation of the ingested agent. SOD = superoxide dismutase, MPO = myeloperoxidase. (After Smith, 1994).

Even more reactive products, such as hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen (O^{2-}) may also be formed, but these products are very short-lived and are of unknown importance (Rosen *et al.,* 1995). The fusion of neutrophil granules with the phagocytic vesicle represents the process of degranulation. This includes the discharge of myeloperoxidase from azurophilic granules which promotes the generation of highly microbicidal, chlorinated oxidants, such as hypochlorous acid (HOCl) and chloramines (Bernofsky, 1991):

$$H_2O_2 + HCl \rightarrow HOCl + H_2O$$

The collective products of respiratory burst activity represent a pool of reactive oxygen species, and together with the contents of neutrophil granules, represent a potent, cytotoxic cocktail with which to ensure efficient killing and degradation of the phagocytosed material.

1.3.8 The Synthesis of Inflammatory Mediators

Activated neutrophils also synthesize and release an important array of additional inflammatory mediators, including PAF (De Nichilo *et al.,* 1991; Camussi *et al.,* 1987), TNFα (Dubravek *et al.,* 1990), IL-8 (Bazzoni *et al.,* 1991), LTB_4 (Bozza *et al.,* 1996) and PGE_2 (Bozza *et al.,* 1996). The release of these mediators, either singly or in combination, from large numbers of activated neutrophils at an inflammatory focus allows the fine tuning of inflammatory responses.

1.4 Neutrophil-Mediated Host Tissue Damage and its Prevention

Since activated neutrophils can generate such highly cytotoxic products, these must be prevented from damaging normal host tissues. Endothelial cells generate substances, including PGI_2 (Zimmerman *et al.,* 1985), which inhibit neutrophil adhesion, and contain "ecto-enzymes" that can convert ATP and ADP into

adenosine, which also inhibits various neutrophil functions (Firestein *et al.,* 1995). The intracellular hypochlorous acid of neutrophils can react with, and thus denature, azurophilic granule enzymes and the NADPH oxidase, in the absence of other substrates (Weiss, 1989). Products released from neutrophils (e.g. during phagocytosis) can be inactivated by various anti-proteases (e.g. α_1-anti-trypsin) and anti-oxidants present in exudate fluids. The final line of host protection is another professional phagocyte, the macrophage, which can recognize and ingest senescent neutrophils prior to the onset of necrosis (see 1.5), thereby preventing extracellular release of cytotoxic products (Savill *et al.,* 1989).

However, if the host's anti-inflammatory control mechanisms are overwhelmed, the microbicidal potential of neutrophils will be turned upon the host, eliciting inadvertent host tissue damage. For example, the pathology of emphysema (Gadek, 1992) has been linked to the deficiency of α_1-anti-trypsin. Host tissue damage will also occur if the specific arms of the immune response (e.g. antibodies and other cells with cytotoxic potential) fail to differentiate between foreign and self antigens, thus marking host tissues for destruction. As a consequence of the above, neutrophils have been implicated in the pathogenesis of a variety of clinical disorders, including the adult respiratory distress syndrome (ARDS) (Wardle *et al.,* 1992), ischaemia-reperfusion injury (Williams, 1994), pulmonary fibrosis (Behr *et al.,* 1991), rheumatoid arthritis (Robinson *et al.,* 1992) and vasculitic diseases (Thomas *et al.,* 1988; Savage and Rees, 1994).

1.5 Neutrophil Apoptosis

Senescent neutrophils die by the process of apoptosis (programmed cell death). This involves cell shrinkage, nuclear condensation and the formation of cytoplasmic blebs (Arends and Wyllie, 1991). Subsequently, the neutrophil undergoes fragmentation into several membrane-bound, apoptotic bodies which are then rapidly removed by mononuclear phagocytes (Savill *et al.,* 1989), a process that fails to elicit an

inflammatory response (Meagher *et al.,* 1992): apoptosis thus represents a means of terminating neutrophil-mediated inflammation.

1.6 Neutrophil Priming

The primary role of the immune system is to defend the host from injurious agents. However, as discussed above, the enormous cytotoxic potential required for such a function predisposes the host to inadvertent tissue damage. Thus, a system has evolved whereby, under normal conditions, circulating immune cells remain in a quiescent and functionally inactive state, until they receive a series of specific activation (and often co-activation) signals. However, it has since been realized that neutrophil behaviour is regulated in a far more complex manner and is highly influenced by environmental factors. Indeed, previous *in vitro* studies have demonstrated that if neutrophils are pre-exposed to certain agents, which themselves fail to activate these cells, their subsequent microbicidal response to a subsequent agonist challenge may be markedly enhanced. This functional up-regulation has been termed *priming*. Neutrophil priming was originally demonstrated with LPS, which did not elicit a respiratory burst alone, but greatly enhanced respiratory burst activity (specifically superoxide anion release) to a subsequent (initially non-activating) challenge with fMLP (Guthrie *et al.,* 1984; Cohn and Morse, 1960). The enhancement of fMLP-stimulated superoxide anion release has since been considered as the "gold standard" *in vitro* rmeasure of neutrophil priming. Although, the definition of neutrophil priming has evolved to include the enhancement of various other functional responses, it still requires that the following criteria be fulfilled:

(i) the priming agent, at its designated concentration, does not initiate the effector function

(ii) the priming agent must precede the activating stimulus.

i.e. **Quiescent neutrophil → Stimulated neutrophil**

(little response)

Quiescent neutrophil → Primed neutrophil → Primed-Stimulated neutrophil

(little response) (major response)

Although *dedicated priming agents* are unable to activate neutrophils directly, irrespective of the concentration used (Hallett and Lloyds, 1995), it is now recognised that other agents may prime neutrophils at low concentrations and activate them at higher concentrations. For instance, low concentrations of fMLP and PMA, both well-established neutrophil stimulants, have been reported to prime responses to a second agonist (Bender and Van Epps, 1983; English *et al.,* 1981; Bellavite *et al.,* 1993; Wymann *et al.,* 1987). These observations imply that priming and activation are closely linked events, which may occur sequentially *in vivo* when neutrophils move along a chemotactic gradient. Furthermore, the demonstration that individual pro-inflammatory mediators may prime one response (e.g. respiratory burst activity) whilst simultaneously "activating" another (e.g. shape change (Haslett *et al.,* 1985) or adhesion molecule up-regulation (Condliffe *et al.,* 1996)), again highlights the difficulty in defining the boundaries between neutrophil priming and activation.

Nevertheless, the current weight of *in vitro* and *in vivo* data indicates that neutrophils will not express their full microbicidal potential to patho-physiological activating agents unless they have first been primed. For example, when neutrophils are incubated with fMLP, the extent of superoxide anion release is directly proportional to their level of priming. This problem has been a major confounding variable in many *in vitro* neutrophil studies, due to the endogenous priming (by agents such as LPS) that is inherent to many neutrophil isolation procedures (Haslett *et al.,* 1985). Thus, it is possible that neutrophils cannot produce a respiratory burst upon activation unless they have first been primed (Pabst, 1994). However, it remains

unclear whether priming represents an increased response of the whole neutrophil population, or whether it symbolizes the recruitment of previously quiescent neutrophils to a responsive state (Hallett and Lloyds, 1995) (see 1.6.3).

1.6.1 Indices of Neutrophil Priming

Since individual agents can induce a range of functional responses in neutrophils, no single test will provide an adequate screen for neutrophil priming. However, if several functions are measured in parallel, this provides a global assessment of neutrophil responses to a particular agent. The respiratory burst is a characteristic property of the professional phagocyte. Thus, the enhancement of respiratory burst activity to a subsequent stimulus remains the most conventional indicator of neutrophil priming. As mentioned above, the gold standard for determining whether neutrophils are primed is taken as the measurement of fMLP-stimulated superoxide anion release, because it relates directly to the microbicidal potential of neutrophils, whilst being a sensitive, objective assay. However, other secondary responses can also be measured in primed neutrophils (Figure 1.4). For example, neutrophils can be primed for degranulation (Fittschen *et al.,* 1988) and enhanced release of various inflammatory mediators, including LTB_4, AA, PGE_2 and PAF (Daniels *et al.,* 1992; Bozza *et al.,* 1996; Doerfler *et al.,* 1994; Doerfler *et al.,* 1989).

The quantification of cellular shape change provides a direct measurement of the chemotactic and chemokinetic potential of neutrophils (Haston and Shields, 1985). Although the intracellular signalling pathways for chemotaxis are distinct from those leading to superoxide anion production (Yasui *et al.,* 1994; Reibman *et al.,* 1991), the degree of neutrophil shape change has been shown to correlate directly with the priming of fMLP-stimulated superoxide anion release (Haslett *et al.,* 1985). Thus, the assessment of neutrophil shape change may provide another sensitive indicator of priming status.

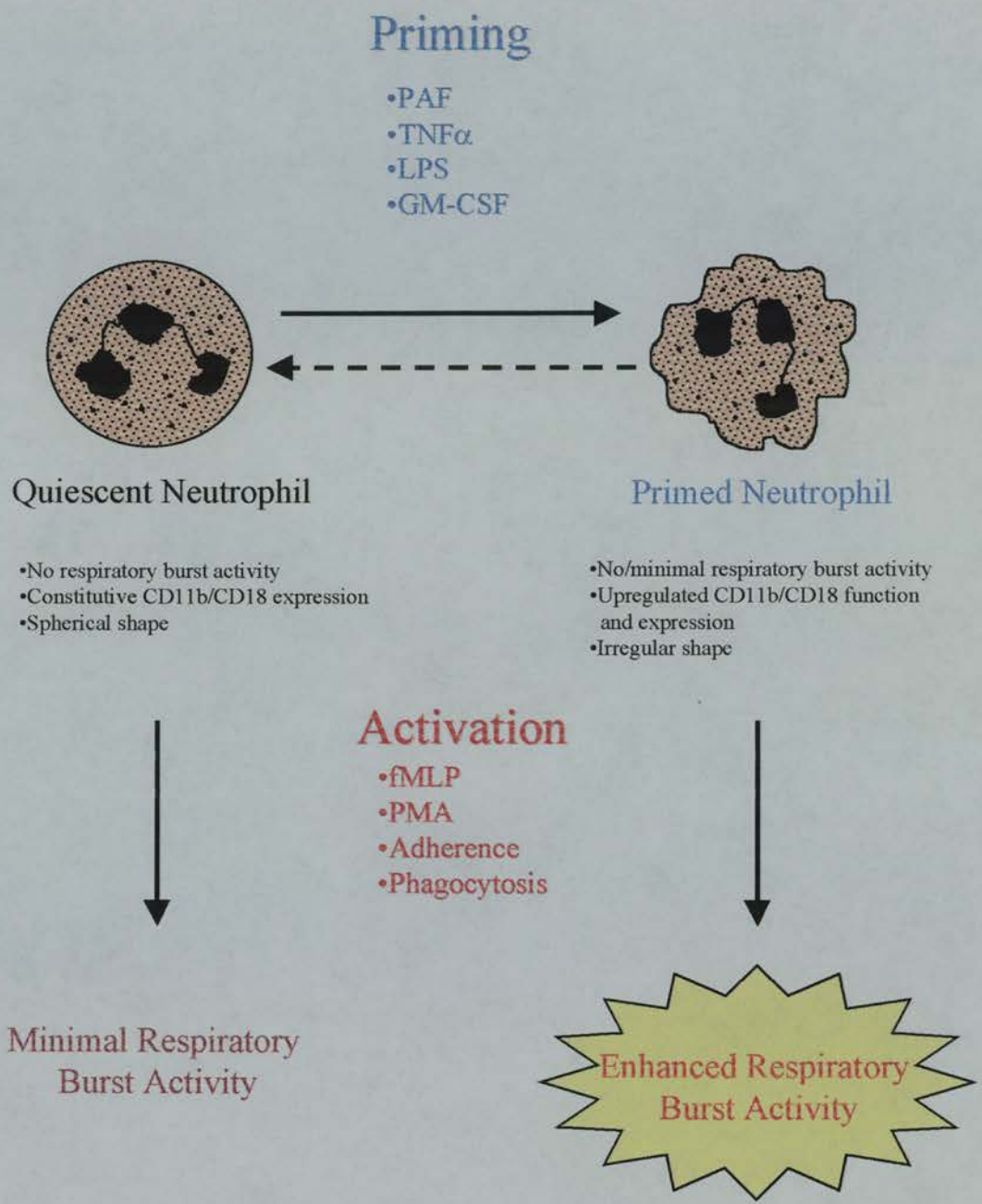


Figure 1.4
Schematic Representation of Neutrophil Priming.
Various pro-inflammatory mediators can prime neutrophils for an enhanced respiratory burst to secretagogue agonists. Priming is associated with other increased functional responses, including neutrophil shape change, adhesiveness, and release of inflammatory mediators.

Neutrophil shape change does not require adhesion (Anderson *et al.,* 1986; Anderson *et al.,* 1985). However, the chemotactic response of neutrophils *in vivo* is intimately associated with a sequence of adhesive events (Jutila *et al.,* 1989; Arfors *et al.,* 1987; Wallis *et al.,* 1986) mediated by changes in selectin and integrin molecule expression and function. It has been reported that cross-linking of L-selectin molecules with monoclonal antibodies, or cell adhesion to glass or plastic surfaces, leads to neutrophil priming (Waddell *et al.,* 1994). In addition, concentrations of stimuli that prime respiratory burst activity have also been shown to alter the relative distribution of neutrophil adhesion molecules, inducing the loss of L-selectin and the up-regulation of CD11b/CD18 expression and avidity (Condliffe *et al.,* 1996; Borregaard *et al.,* 1994; Griffin *et al.,* 1990; Kishimoto *et al.,* 1989). Thus, neutrophil priming may be concerned with the recruitment of "rolling" neutrophils to an inflammatory site.

The following model for the role of neutrophil priming in the inflammatory response can therefore be proposed: during an acute inflammatory response *in vivo*, priming may occur (in response to soluble or cell-associated priming agents) as neutrophils marginate or roll along the endothelial surface. These neutrophils still express L-selectin and are exposed to cytokines and low concentrations of chemotactic factors. The subsequent exocytosis of secretory vesicles results in the incorporation of vesicle membrane, rich in CD11b/CD18, but devoid of L-selectin (Borregaard *et al.,* 1994), into the plasma membrane, whilst inducing shape change. The up-regulation of β_2-integrins and shedding of L-selectin promotes the endothelial arrest of primed neutrophils. During their ensuing trans-endothelial migration, increasing concentrations of chemotactic factors provide increasing neutrophil stimulation, leading to more pronounced cell polarization and granule exocytosis, with the continuing up-regulation of plasma membrane components (e.g. receptors for fMLP and the opsonins, and cytochrome b_{558}). When these functionally up-regulated neutrophils have reached the inflamed site, the high concentrations of soluble stimuli and opsonised particles then triggers full activation, initiating phagocytosis,

degranulation and respiratory burst activity. Thus, if neutrophils have been primed for action *en route*, they will hopefully achieve their full microbicidal potential.

1.6.2 Neutrophil Priming Agents

Many conditions have been reported to prime neutrophils. Nevertheless, priming agents can be broadly classified as physiological, physico-chemical, or pharmacological. Although studies with physiological mediators have the most relevance to *in vivo* events, much can be learned from the artificial manipulation of priming with the latter two groups of agents. Indeed, the recognition that venepuncture itself may (under certain circumstances) result in neutrophil priming, illustrates the importance of maintaining neutrophil homeostasis at all times, especially during *ex vivo* priming studies.

Physiological priming agents act through specific, cell-surface receptors, of which there are at least three different classes. The classic, G-protein-linked receptors, consisting of seven transmembrane domains, mediate the effects of the majority of neutrophil chemoattractants: thus, fMLP, PAF, C5a, IL-8, and substance P all utilize this family of receptors (Gerard and Gerard, 1991; Murphy and Tiffany, 1991; Boulay *et al.,* 1990). The cytokine/growth factor family of receptors comprise a single transmembrane domain, and signal the effects of agents such as TNFα, GM-CSF and G-CSF (Tartaglia and Goeddel, 1992). The third group of receptors also exhibit a single transmembrane domain, but require immobilization or crosslinking (rather than ligand occupancy) for activation: this group includes the integrins and FcR family (Metzger, 1992; Ng-Sikorski *et al.,* 1991). The structural heterogeneity of these receptors reflects their different functional roles and may provide insight into their downstream signalling targets. In addition, it may help to explain the variability between the onset and duration of priming induced by different classes of agents.

The following section will introduce the main physiological priming agents investigated in this thesis.

1.6.2.1 Platelet-Activating Factor

Since PAF can activate a wide variety of cell types (Chao and Olson, 1993), it is not surprising that it may contribute to many physiological and patho-physiological processes *in vivo*, including vasodilatation and hypotension (Sun *et al.*, 1990), allergic and inflammatory reactions (Braquet *et al.*, 1987; Humphrey *et al.*, 1982), asthma and transplant rejection (Page, 1990; Foegh, 1988; Vargaftig, 1987; Feuerstein and Hallenbeck, 1987; Braquet *et al.*, 1987). Although platelet-activating factor (PAF) was originally identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (Demopoulos *et al.*, 1979; Benveniste *et al.*, 1979), it is not a single molecular species, but represents a family of molecules with an alkyl linkage at the *sn*-1 position, an *sn*-2 short-chain acyl group, and a polar phosphocholine head group. However, many cells that were originally thought to produce *alkyl*-PAF may also produce *acyl*-PAF, a group of bioactive molecules with an *sn*-1 acyl linkage (Pinckard and Prihoda, 1996; Triggiani *et al.*, 1991; Alonso *et al.*, 1986) that confound the bioassay of PAF.

The majority of cell types produce PAF upon appropriate stimulation, including vascular endothelial cells (Prescott *et al.*, 1984), mast cells (Mencia-Huerta *et al.*, 1983), macrophages (Albert and Snyder, 1983), platelets (Chignard *et al.*, 1980) and neutrophils (Lynch *et al.*, 1979): neutrophils have been reported to generate more PAF than any other inflammatory cell, estimated at 10-100 pmol PAF per 10^6 neutrophils following calcium ionophore treatment (Oda *et al.*, 1985; Jouvin-Marche *et al.*, 1984; Lynch and Henson, 1986). Cell stimulation initiates the remodelling pathway for PAF synthesis, probably in a light membrane fraction such as the endoplasmic reticulum (Riches *et al.*, 1985; Ribbes *et al.*, 1985; Saffitz *et al.*, 1986; Mollinedo *et al.*, 1988). The rate-limiting step in PAF synthesis is the release of arachidonic acid (AA) and lyso-PAF from membrane phospholipids, by the action of phospholipase A_2 (Chilton *et al.*, 1984): lyso-PAF is both a precursor and metabolite of PAF. The *de novo* synthesis of PAF also occurs (Snyder, 1987), but its contribution to the overall levels of PAF remains unclear.

PAF mediates its effects through a seven-transmembrane domain, G-protein-linked receptor (Nakamura *et al.,* 1991). There is some confusion in the literature regarding the possible heterogeneity of these receptors, confounded by repeated attempts to correlate the results of binding studies performed under many different assay conditions. However, virtually all studies agree that: (i) neutrophils contain specific, high affinity PAF receptors (with reported K_D values of 0.1-16.3 nM); (ii) these receptors are localized to the plasma membrane and are responsible for the bioactions of PAF; and (iii) PAF receptor antagonists can inhibit neutrophil responses to PAF (Casals-Stenzel *et al.,* 1987; Shen *et al.,* 1985; O'Flaherty *et al.,* 1989; Dent *et al.,* 1989; O'Flaherty *et al.,* 1986; Marquis *et al.,* 1988; Hwang, 1988; O'Flaherty and Nishihira, 1987). An "earmuffs" model has been proposed for the PAF binding site (Godfroid *et al.,* 1991), with a central hydrophobic pocket (for insertion of the *sn*-1 alkyl/acyl group) embedded below two, opposing zones of positive charge (for interaction with the polar phosphocholine head group). In addition to the extracellular PAF receptor(s), there have also been suggestions that neutrophils contain intracellular PAF binding sites (De Kimpe *et al.,* 1995; Svetlov and Nigam, 1993; Muller and Nigam, 1992), which may mediate some of the autocrine actions of PAF retained within its cell of origin.

The effects of PAF on human neutrophils have been widely documented. *In vitro*, PAF has been shown to induce a variety of functional responses, including aggregation (Camussi *et al.,* 1980), chemotaxis (Pinckard *et al.,* 1992; Shaw *et al.,* 1981), and adherence to endothelial cells (Ingraham *et al.,* 1982). However, PAF does not directly stimulate respiratory burst activity unless neutrophils have been pre-treated with cytochalasin B or propanolol, or PAF is present at high (>10 μM) concentrations (Gay, 1993; Shaw *et al.,* 1981; Pinckard *et al.,* 1992). Instead, PAF is a potent and rapid-acting neutrophil priming agent, enhancing the subsequent respiratory burst induced by activating stimuli such as fMLP, PMA or C5a (Pinckard *et al.,* 1992; Dewald and Baggiolini, 1985; Gay *et al.,* 1986; Ingraham *et al.,* 1982). PAF has also been shown to prime AA mobilization and eicosanoid formation

(Bozza *et al.,* 1996; Tanizawa and Tai, 1989), and (in the presence of cytochalasin B) degranulation (Vercellotti *et al.,* 1988; Shaw *et al.,* 1981; Ingraham *et al.,* 1982).

Although neutrophil priming *in vitro* has been most widely studied with PAF acting in solution, PAF may also function *in vivo* as a cell-associated, pro-inflammatory mediator. It has been proposed that when endothelial cells are stimulated by certain agonists (including thrombin, LTC_4, histamine, bradykinin and ATP) they synthesize PAF rapidly (McIntyre *et al.,* 1985; McIntyre *et al.,* 1986; Prescott *et al.,* 1984) and express this molecule on the cell surface alongside the tethering molecule P-selectin (Lorant *et al.,* 1991). In this way, PAF has been proposed to contribute to the juxtacrine stimulation of rolling neutrophils (Lorant *et al.,* 1993; Lorant *et al.,* 1991), an event which requires the engagement of specific PAF receptors on the neutrophil surface (Vercellotti *et al.,* 1988; Hwang, 1988). According to this model, PAF stimulates up-regulation of both the avidity and surface expression of CD11b/CD18, the shedding of L-selectin, and the firm adherence of neutrophils to the activated endothelium (Lorant *et al.,* 1991; Ingraham *et al.,* 1982). Concurrently, neutrophils become polarized (Lorant *et al.,* 1993) and primed for enhanced respiratory burst activity and degranulation prior to their extravasation (Lorant *et al.,* 1993; Vercellotti *et al.,* 1989).

1.6.2.2 Tumour Necrosis Factor-α

Tumour necrosis factor-α (TNFα) is a cytokine that was originally identified by its cytotoxic activity against tumour cells (Carswell *et al.,* 1975). It has also been demonstrated that TNFα is a mediator of endotoxin-induced septic shock, cachexia (hence its other name, cachectin) and inflammatory responses (Beutler and Cerami, 1989; Beutler and Cerami, 1988). TNFα is produced by activated cells of the immune system, particularly by LPS-exposed macrophages (Aggarwal *et al.,* 1985b). Mature TNFα is cleaved proteolytically from the cell surface by the action of TNFα-converting enzyme (TACE) (Black *et al.,* 1997) and exists as a homotrimeric molecule composed of 17 kDa subunits (Wingfield *et al.,* 1987; Aggarwal *et al.,*

1985). It mediates its effects through two distinct TNF receptor subtypes of 55 and 75 kDa, of which the former is present on the majority of cell types whilst the latter is present only on cells of haemopoeitic lineage (Brockhaus *et al.,* 1990) .

TNFα has various effects upon neutrophil function. It is chemotactic (Figari *et al.,* 1987; Ming *et al.,* 1987) and can increase the surface expression of receptors, including CD11b/CD18 (Ozaki *et al.,* 1988; Berger *et al.,* 1988). It can prime the respiratory burst of neutrophils in suspension to agents such as fMLP and PMA (Elbim *et al.,* 1994; Tennenberg and Solomkin, 1990; Berkow *et al.,* 1987) and is a direct activator of superoxide anion generation in adherent neutrophils (Schleiffenbaum and Fehr, 1990; Nathan, 1987).

1.6.2.3 Lipopolysaccharide

Lipoplysaccharide (LPS or bacterial endotoxin) is a major cell-membrane component of Gram-negative bacteria, which is believed to contribute to the widespread patho-physiology of Gram-negative septicaemia and the pulmonary vascular injury associated with ARDS (Parsons *et al.,* 1989). The LPS molecule is composed of a variable polysaccharide moiety which is expressed externally, linked to an intramembranous lipid A component, comprising a highly-conserved, diglucosamine-based phospholipid (Kulshin *et al.,* 1992) which appears to contain the bioactive portion of LPS (Galanos *et al.,* 1985). LPS can elicit neutrophil shape change, aggregation and adhesion molecule up-regulation (Dahinden *et al.,* 1983). In addition, LPS is a well-recognized neutrophil priming agent that has been reported to enhance respiratory burst activity and LTB_4 production in response to a second stimulus such as fMLP, PMA or immune complexes (Aida and Pabst, 1990; Doerfler *et al.,* 1989; Guthrie *et al.,* 1984).

Several studies have suggested that the effects of LPS on neutrophils are mediated by CD14, a membrane glycoprotein (Shapira *et al.,* 1995; Weingarten *et al.,* 1993) which is believed to function as its cell-surface receptor. However, it has been

demonstrated that the response of neutrophils to LPS is greatly enhanced in the presence of plasma (Aida and Pabst, 1990): an acute phase protein, named lipopolysaccharide binding protein (LBP), was proposed to mediate this facilitation (Vosbeck *et al.,* 1990; Tobias *et al.,* 1986). Thus, it is currently believed that LPS forms a complex with LBP, and that this complex then primes neutrophils via CD14 (Shapira *et al.,* 1995; Yasui *et al.,* 1992; Vosbeck *et al.,* 1990; Wright *et al.,* 1991). Other serum LPS-binding proteins, the septins, have also been identified (Wright *et al.,* 1992), but are not thought to contribute to the priming effects of LPS (Shapira *et al.,* 1995).

1.6.2.4 Inositol Hexakisphosphate

Inositol hexakisphosphate ($InsP_6$) is the most abundant inositol phosphate found in nature (Cosgrove, 1980), being found in mammalian cells at concentrations between 10 μM and 1 mM (Szwergold *et al.,* 1987). It is an intriguing molecule whose true physiological role has yet to be revealed. However, $InsP_6$ has been proposed to have various intracellular functions, for example, acting as a general antioxidant (Graf and Eaton, 1990), Ca^{2+} chelator (Luttrell, 1993), inhibitor of iron-catalysed hydroxyl radical formation (Hawkins *et al.,* 1993) and phosphate store (Berridge and Irvine, 1989).

$InsP_6$ may also have a number of extracellular actions: it has been reported to lower blood pressure and heart rate when infused into specific regions of the rat brain-stem (Vallejo *et al.,* 1987). $InsP_6$ can also suppress the development of colonic cancer in animal models, probably by chelating metal ions and thereby limiting mitogenic, iron-catalysed, redox reactions (Graf and Eaton, 1993). At a cellular level, $InsP_6$ has also been shown to elicit Ca^{2+} influx and catecholamine release in bovine adrenal chromaffin cells (Regunathan *et al.,* 1992) and to enhance Ca^{2+} influx in cultured neuronal cells (Nicoletti *et al.,* 1989).

However, in addition to these diverse actions, it has also been suggested that $InsP_6$ may have a pro-inflammatory role by acting as a neutrophil priming agent (Eggleton *et al.,* 1991), an action not observed with other inositol polyphosphates. It was reported that the preincubation of human neutrophils with $InsP_6$ led to an enhanced superoxide anion generation to fMLP (Eggleton *et al.,* 1991) and a rapid, yet sustained, assembly of F-actin (Crawford and Eggleton, 1992). Thus it has been proposed that effete cells present at an inflammatory focus might release $InsP_6$, which could then function to prime adjacent neutrophils and thereby augment the inflammatory response.

1.6.2.5 Adenosine Triphosphate

Adenosine triphosphate (ATP) is released from a variety of cell types by a process of exocytosis. The stimulated release of ATP has been particularly well studied in the context of platelet aggregation, where it has been estimated that the local, extracellular concentration of ATP may reach 12 μM (Ingerman *et al.,* 1979). Such elevated levels of ATP may be transient however because endothelial cells possess ecto-nucleotidases that can rapidly hydrolyze ATP to adenosine, via ADP and AMP (Pearson *et al.,* 1980),

Extracellular ATP has been reported to activate a variety of cell types (Hallam and Pearson, 1986) via P_2 purinoceptors (Burnstock, 1978). Furthermore, ATP has been shown to elicit rapid neutrophil priming for enhanced superoxide anion generation to both fMLP and immune complexes (Naum *et al.,* 1991; Ward *et al.,* 1988; Kuhns *et al.,* 1988): this priming effect was associated with an influx of Ca^{2+} (Kuhns *et al.,* 1988). Thus, it was postulated that ATP released from stimulated platelets *in vivo* might modulate the functions of nearby neutrophils (Kuhns *et al.,* 1988). Nevertheless, these pro-inflammatory effects of ATP might be limited by its subsequent metabolism to adenosine, an inhibitor of various inflammatory responses which signals through several distinct receptor subtypes (Walker *et al.,* 1990; Cronstein *et al.,* 1983).

1.6.3 Neutrophil Priming *in Vivo*

Neutrophil priming was initially described as an *in vitro* phenomenon. However, since the majority of priming agents are established pro-inflammatory mediators released during various patho-physiological states, it is hardly surprising that there are numerous examples of priming occurring *in vivo*.

Primed neutrophils have been reported in the blood of patients with Hodgkin's disease (Tullgren *et al.,* 1991), psoriasis (Bloomfield and Young, 1988), inflammatory bowel disease (Suematsu *et al.,* 1987), ARDS (Chollet-Martin *et al.,* 1992), sarcoidosis (Barth *et al.,* 1988), bacterial and fungal infections (Bass *et al.,* 1986), and essential hypertension (Pontremoli *et al.,* 1989). In addition, neutrophil priming is apparent in the blood of otherwise healthy individuals following blunt trauma (Krause *et al.,* 1988) and moderate exercise (Smith *et al.,* 1990), and primed neutrophils have been isolated from the joints of people with active rheumatoid arthritis (Robinson *et al.,* 1992). Evidence of neutrophil activation (as indicated by an increased plasma concentration of neutrophil granule contents) has been observed in septicaemia (Panyutich *et al.,* 1993), bacterial meningitis (Panyutich *et al.,* 1993) and following strenuous exercise (Dufaux and Order, 1989; Pyne, 1994).

The mechanisms involved in neutrophil priming are unknown in the majority of cases, because detectable levels of circulating cytokines are only found in extreme circumstances. Nevertheless, the priming observed in septicaemia and ARDS have been found to correlate with circulating levels of TNFα (Chollet-Martin *et al.,* 1993; Chollet-Martin *et al.,* 1992; Trautinger *et al.,* 1991). Indeed, persistently high levels of TNFα and IL-6 have been linked to a poor outcome in septic shock (Pinsky *et al.,* 1993). Similarly, persistent endotoxaemia (circulating LPS) has been associated with the development of ARDS (Parsons *et al.,* 1989).

It is important to mention at this stage, that peripheral blood neutrophils are not a homogeneous population; instead, they can be divided into *sub-populations* of

different age and morphology on the basis of cell-surface markers. Furthermore, functional heterogeneity has been reported within the circulating neutrophil pool (Daniels *et al.,* 1994; Bass *et al.,* 1986; Klempner and Gallin, 1978). Although the physiological significance of functional heterogeneity is not fully understood, it may indicate the previous history (e.g. margination) of circulating neutrophils. The distribution of neutrophil sub-populations in peripheral blood is affected by various patho-physiological states, including inflammatory disease, infection and exercise; for example, patients with ARDS or acute bacterial infections have a sub-population of peripheral blood neutrophils that show enhanced responses for respiratory burst activity (Bass *et al.,* 1986; Chollet-Martin *et al.,* 1992) and antibody-mediated phagocytosis (Simms *et al.,* 1989). Although young neutrophils are released from the bone marrow in response to infection (Simms *et al.,* 1989), the number of juvenile cells appears to be unrelated to the proportion of primed neutrophils and their oxidative potential (Bass *et al.,* 1986). Thus, the appearance of distinct neutrophil sub-populations in response to priming stimuli, may indicate that priming recruits previously non-responsive cells to a responsive state, rather than augmenting the responses of all neutrophils (Daniels *et al.,* 1994).

1.7 Potential Mechanisms of Neutrophil Priming

Although the mechanisms underlying neutrophil *activation* have been widely studied, the process of *priming* has received less attention. Progress in this area is hampered by the complex, and divergent, intracellular signalling pathways utilized by different neutrophil stimulants. However, the close links between the primed and activated state suggest that many of the mechanisms described for neutrophil activation may also be relevant to priming (Figure 1.5). Thus, the following section will outline current knowledge regarding potential mechanisms that have been proposed to mediate neutrophil priming. In broad terms, previous data have suggested that priming agents may up-regulate secretagogue-mediated responses at the receptor, G-

protein or cytosolic second messenger level, but a universally applicable mechanistic basis for neutrophil priming has yet to be established.

1.7.1 fMLP Receptors

The exposure of neutrophils to various priming agents, including PAF, TNFα and LPS, and low concentrations of C5a and fMLP, has been associated with a Ca^{2+}-dependent up-regulation of fMLP receptor expression (Vosbeck *et al.,* 1990; Goldman *et al.,* 1986; Tennenberg and Solomkin, 1990; Norgauer *et al.,* 1991). This has been proposed to result from the exocytosis of specific granules and secretory vesicles, and occurs: (i) independently of alterations in receptor affinity (Tennenberg and Solomkin, 1990) and (ii) in concert with CD11b/CD18 (CR3) up-regulation (Norgauer *et al.,* 1991). Thus, it was suggested that an increased expression of fMLP receptors in primed neutrophils might explain the enhanced responses observed upon subsequent exposure to fMLP. However, it has been demonstrated that for both TNFα-induced priming of degranulation (O'Flaherty *et al.,* 1991) and calcium ionophore-induced priming of respiratory burst activity (Andersson *et al.,* 1987), the enhanced functional response precedes the increase in fMLP binding. Furthermore, the priming of respiratory burst activity elicited by IL-8 occurs at concentrations below those that result in a detectable increase in fMLP receptor number (Roberts *et al.,* 1993). Thus, the up-regulation of fMLP receptors is unlikely to underlie the priming of subsequent responses to fMLP.

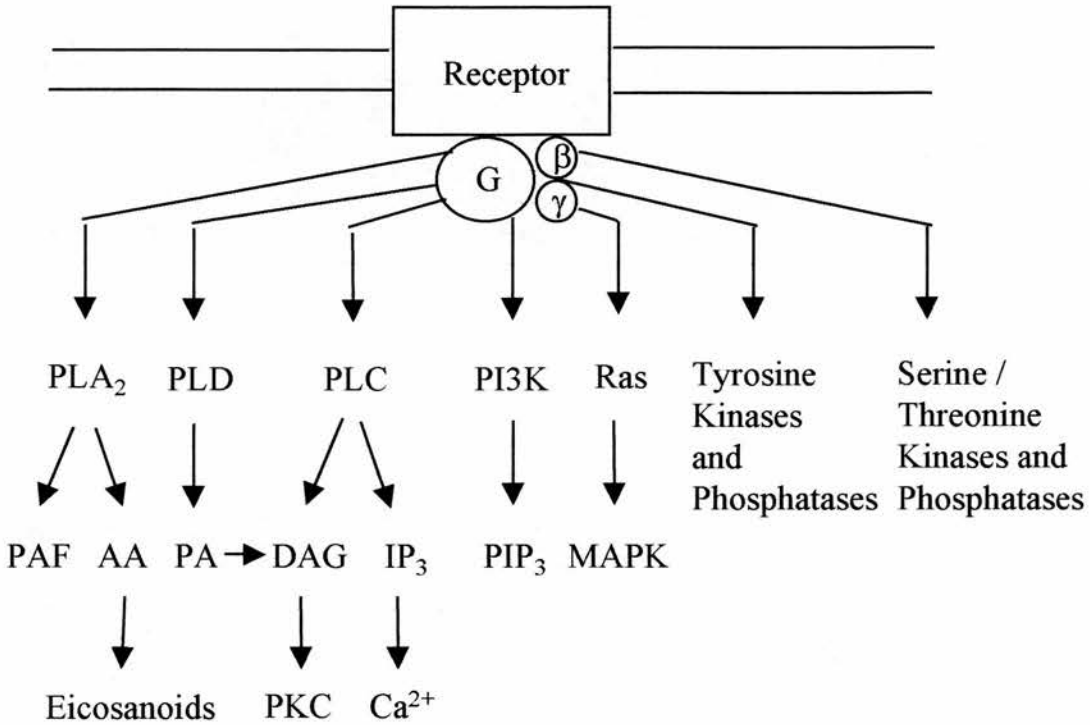


Figure 1.5
Schematic Diagram of Multiple Signalling Pathways Triggered by Activation of G-protein-linked Receptors in Neutrophils.

1.7.2 G-Proteins

Intracellular proteins (either cytosolic or membrane-associated) which bind GTP are known as G-proteins. G-proteins are broadly classified as either heterotrimeric or small molecular weight (approximately 20-25-kDa) monomeric G-proteins. Heterotrimeric G-proteins are composed of α-and βγ-subunits, and regulate cellular events by a GTP hydrolysis cycle: the replacement of GDP (bound to the α-subunit) with GTP results in the dissociation of α- and βγ-subunits, and G-protein activation (Neer and Clapham, 1988). Although the activated α-subunit is thought to be the principal effector molecule in the majority of circumstances, free βγ-subunits can also initiate certain responses (Lee and Rhee, 1995; Katz *et al.,* 1992). The intrinsic GTPase activity of the α-subunit subsequently hydrolyses GTP to GDP, whereby α and βγ recombine to end the activation cycle.

Heterotrimeric G-proteins couple the seven-transmembrane-domain family of receptors utilized by the majority of neutrophil chemoattractrants (Neer, 1995) to intracellular systems such as actin polymerization (Bengtsson, 1990) and the phospholipases-C, -D and -A_2 (Taylor, 1990). Although the identity of the βγ-subunits expressed in neutrophils are unknown, many different α-subunits have been identified, including members of the $G_{s\alpha}$, $G_{i\alpha}$, and $G_{q\alpha}$ classes (Thelen *et al.,* 1993): however, the major α-subunits in human neutrophils are $G_{i\alpha2}$ and $G_{i\alpha3}$ (Goldsmith *et al.,* 1987).

Both $G_{i\alpha2}$ and $G_{i\alpha3}$ are sensitive to pertussis toxin, which ADP-ribosylates and inactivates them, thereby uncoupling these proteins from receptors. Since pertussis toxin inhibits the superoxide response to fMLP (Snyderman *et al.,* 1986; Lad *et al.,* 1985; Gay, 1993), other neutrophil stimulants which do not utilize G-protein-linked receptors (e.g. PMA and calcium ionophores such as A23187) have been employed to investigate the influence of pertussis toxin on neutrophil priming. However, the literature regarding the pertussis toxin sensitivity of neutrophil priming is confusing. For example, whilst it has been reported that pertussis toxin does not affect TNFα-

induced (Berkow and Dodson, 1988) or PAF-induced (Gay, 1993) priming of the superoxide response to PMA, nor the priming of A23187-induced AA release by GM-CSF (Di Persio *et al.,* 1988; Di Persio and Abbound, 1992), another study found that pertussis toxin abolished GM-CSF priming of A23187-induced AA release (McColl *et al.,* 1989).

Nevertheless, there is preliminary evidence to suggest that priming agents, including LPS (Yasui *et al.,* 1992), GM-CSF (Durstin *et al.,* 1993) and TNFα (Klein *et al.,* 1995), elicit the translocation of $G_{i\alpha2}$ to the neutrophil membrane. Since G-protein translocation increases the pool size of relevant G-proteins available for chemoattractant receptor-mediated signal transduction, this event may contribute to the potentiation of chemoattractant-induced responses. Indeed, the time-courses for the priming of fMLP-stimulated superoxide anion release by LPS, PAF and TNFα, have been shown to correlate with the translocation of $G_{i\alpha2}$ to the plasma membrane (Alison Condliffe, personal communication). This may also be the case with GM-CSF, although the translocation of $G_{i\alpha2}$ to the plasma membrane was observed to be more rapid (Durstin *et al.,* 1993) than its superoxide-priming effect (Weisbart *et al.,* 1986). However, the exact role of G-proteins in GM-CSF signalling remains somewhat controversial (Durstin and Sha'afi, 1996; Probst *et al.,* 1992). Thus, (certain) priming agents may promote the association of α-subunits with the plasma membrane, which may contribute to their facilitation of subsequent responses.

1.7.3 Phospholipase C and Ins(1,4,5)P_3

Human neutrophils contain phosphoinositide-specific phospholipase C (PLC), an enzyme responsible for the cleavage of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and *sn*1,2-diacylglycerol (DAG) (Thelen and Wirthmueller, 1994). Neutrophil stimulation, either with chemotactic factors (e.g. fMLP, LTB_4, C5a, PAF, IL-8) or via phagocytic receptors (CR3, FcγR), results in the activation of the β and γ isoforms of PLC, respectively. This results in the rapid, but transient, accumulation of Ins(1,4,5)P_3, and a similar

rapid elevation of DAG. Ins(1,4,5)P_3 signals the release of Ca^{2+} from intracellular stores, and DAG is an activator of PKC. There has been limited study into a potential role for Ins(1,4,5)P_3 in neutrophil priming. In the few studies of GM-CSF-induced priming where this has been examined, it was reported that GM-CSF did not affect (Bourgoin *et al.,* 1990; Corey and Rosoff, 1989) basal or stimulated levels of Ins(1,4,5)P_3. This area of research requires further investigation.

1.7.4 Intracellular calcium

The intracellular concentration of free calcium ($[Ca^{2+}]_i$) is maintained at low levels (approximately 100 nM) in resting neutrophils (Sklar *et al.,* 1985). Neutrophil stimulation leads to a biphasic increase in $[Ca^{2+}]_i$, with a rapid Ca^{2+} spike resulting from the mobilization of intracellular Ca^{2+} stores by Ins(1,4,5)P_3, followed by a more sustained phase of Ca^{2+} influx; the emptying of intracellular stores regulates the permeability of the plasma membrane to Ca^{2+} thereby increasing Ca^{2+} influx (Demaurex *et al.,* 1994; Putney, 1986).

The elevation of $[Ca^{2+}]_i$ contributes to neutrophil shape change, degranulation, and superoxide anion generation (Morel *et al.,* 1991; Richter *et al.,* 1990; Pozzan *et al.,* 1988). For example, it has been demonstrated that a threshold $[Ca^{2+}]_i$ of 250 nM is required to elicit a respiratory burst (Hallett *et al.,* 1990), and that depletion of extracellular Ca^{2+} can prevent respiratory burst activation (Sha'afi *et al.,* 1988; Dewald *et al.,* 1988). However, Ca^{2+} oscillations, rather than sustained elevation, may control certain neutrophil functions, including those arising from β_2-integrin activation (Hendey *et al.,* 1992; Jaconi *et al.,* 1991), and Ca^{2+}-independent pathways of neutrophil activation may also exist (Gomez-Cambronero *et al.,* 1989; Rossi *et al.,* 1988; Morel *et al.,* 1991). It is important to note that neutrophil activation causes alterations in the concentration of other ions aside from Ca^{2+}, including a transient reduction in pH_i, and a subsequent increase in $[Na^+]_i$ resulting from activation of the Na^+/H^+ antiport (Weisman *et al.,* 1987).

The role of $[Ca^{2+}]_i$ in priming is unclear. Although certain priming agents such as PAF (Ingraham *et al.,* 1982), ATP (Kuhns *et al.,* 1988) and calcium ionophores (e.g. ionomycin) (Finkel *et al.,* 1987) can elicit Ca^{2+} mobilization, in general the increased $[Ca^{2+}]_i$ has returned to baseline well before the addition of the second activating agonist (Grynkiewicz *et al.,* 1985; Ward *et al.,* 1988; Vercellotti *et al.,* 1988; Ingraham *et al.,* 1982). Furthermore, PAF has been shown to prime fMLP-stimulated superoxide anion release under $[Ca^{2+}]_i$-buffered conditions (Gay, 1993; Walker *et al.,* 1991; Koenderman *et al.,* 1989). TNFα (Yuo *et al.,* 1989; Klein *et al.,* 1990; Lloyds *et al.,* 1995) and substance P (Lloyds *et al.,* 1993) do not mobilize Ca^{2+} or augment the calcium transient elicited by other agonists, and the literature regarding the actions of LPS (Klein *et al.,* 1990; Yee and Christou, 1993; Doerfler *et al.,* 1989; Forehand *et al.,* 1989) and GM-CSF (McColl *et al.,* 1991; Sullivan *et al.,* 1987) is confusing. Thus, it would appear that an elevation in $[Ca^{2+}]_i$, although elicited by certain priming agents, is not an essential component of neutrophil priming.

1.7.5 Phospholipase D and Phosphatidic Acid

Phospholipase D (PLD) mediates the cleavage of phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline. Although the activation pathway for PLD in neutrophils is not as well understood as that of PLC, a wide variety of agents have been shown to activate PLD in neutrophils, including PAF, fMLP, ATP, LTB_4, C5a, C3b and C3bi (Fallman *et al.,* 1993; Thompson *et al.,* 1991).

PA may be involved in activaton of the NADPH oxidase (Bonser *et al.,* 1989; Morel *et al.,* 1991) and actin polymerization (Ha and Exton, 1993), and has also been proposed to play a role in neutrophil priming. For example, it has been shown that fMLP-induced respiratory burst activity in neutrophils does not require PLD activation unless the cells have first been primed (Watson *et al.,* 1994). In addition, although neutrophil priming with TNFα (Bauldry *et al.,* 1991) or GM-CSF (Bourgoin *et al.,* 1990) has no significant effect upon basal levels of PA, it leads to

an increased, sustained accumulation of PA following fMLP stimulation. Since the subsequent conversion of PA to DAG by phosphatidate phosphohydrolase is believed to elicit the delayed secondary phase of DAG accumulation that is observed following fMLP stimulation (Billah *et al.,* 1989; Truett *et al.,* 1988), this may also contribute to neutrophil priming (see below).

1.7.6 Diacylglycerol and Protein kinase C

Neutrophils contain multiple isoforms of the protein kinase C (PKC) family of serine-threonine kinases, the most abundant being the Ca^{2+}-dependent PKCβ (Majumdar *et al.,* 1993; Majumdar *et al.,* 1991). PKC has been shown to translocate to the plasma membrane within seconds of neutrophil stimulation (Christiansen, 1988). Activation of PKCα or PKCβ results from either an increase in intracellular DAG alone, or the synergistic action of Ca^{2+} and DAG (Lee and Bell, 1991). In addition, phorbol esters (e.g. PMA) can bind directly to, and thereby activate, PKC. PKC activation induces the phosphorylation of various neutrophil proteins, including G-actin, myosin light chain, profilin, and $p47^{phox}$: this results in the stimulation of many neutrophil responses such as superoxide anion generation, PLD activity and specific granule exocytosis, and the inhibition of others, including chemotaxis and Ca^{2+} mobilization (Faust *et al.,* 1995; El Benna *et al.,* 1994; Naccache, 1985).

The use of PMA and synthetic DAG has provided evidence that PKC may play a key role in activation of the respiratory burst (Robinson *et al.,* 1985). The ability of sub-activating concentrations of PMA to prime neutrophils also suggests a possible role for PKC in neutrophil priming. PAF stimulation has been reported to increase PKC activity in the particulate fraction of human neutrophils (Gay and Stitt, 1988a) and to enhance that induced by fMLP or PMA (Gay and Stitt, 1988b). In addition, PKC inhibitors (sphinganine and staurosporine) have been shown to abolish PAF-induced priming of superoxide anion generation (Gay, 1993). However, other studies have shown that priming induced by TNFα (Berkow and Dodson, 1988), LPS (Forehand *et al.,* 1989) and GM-CSF (Sullivan *et al.,* 1987) was not associated with prior

translocation of PKC to the neutrophil plasma membrane, and that TNFα fails to either elicit DAG formation directly or enhance that induced by fMLP (Bauldry *et al.,* 1991). In contrast, GM-CSF has been reported to augment the DAG response to fMLP (Bourgoin *et al.,* 1990; Tyagi *et al.,* 1989). Therefore, the contribution of DAG and PKC to neutrophil priming may be dependent upon the particular agent used.

1.7.7 Phosphoinositide 3-OH kinase

Phosphoinositide 3-OH kinase (PI3K) is a ubiquitous enzyme that catalyses the conversion of PtdIns(4,5)P_2 to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). Although the majority of cells express the p110-p85 heterodimeric form of PI3K, there is evidence that neutrophils and platelets contain an additional isoform of PI3K that is not associated with the 85-kDa subunit and is activated by βγ-subunits of G-proteins (Stephens *et al.,* 1997; Stephens *et al.,* 1994). PI3K has been implicated in various intracellular signalling pathways (Vlahos *et al.,* 1995), including cytoskeletal events such as membrane ruffling (Malarkey *et al.,* 1995). Furthermore, the demonstration that the fungal metabolite wortmannin (a specific and irreversible inhibitor of PI3K when used at low concentrations) can abolish the respiratory burst elicited by fMLP or opsonised particles (Ahmed *et al.,* 1995; Arcaro and Wymann, 1993; Dewald *et al.,* 1988; Baggiolini *et al.,* 1987), has implicated PI3K as an obligate component of the signalling pathway for superoxide anion generation (Stephens *et al.,* 1993a; Traynor-Kaplan *et al.,* 1989a).

Although many neutrophil agonists have been shown to activate PI3K (Traynor-Kaplan *et al.,* 1989), its role in priming remains uncertain. Several priming agents, including GM-CSF (Corey *et al.,* 1993), PAF and ATP (Stephens *et al.,* 1993a; Stephens *et al.,* 1993a), have been reported to elicit the accumulation of PtdIns(3,4,5)P_3 in neutrophils. However, whilst TNFα alone does not affect PtdIns(3,4,5)P_3 accumulation (Corey *et al.,* 1993), recently it has been shown to enhance the accumulation induced by fMLP (Alison Condliffe, personal

communication). Thus, augmentation of secretagogue-induced PI3K activation may be important in the priming of human neutrophils. Since PI3K may signal through the small G-proteins $p21^{ras}$ (Rodriguez-Viciana *et al.,* 1994) and $p21^{rac}$ (Hawkins *et al.,* 1995) this also suggests a downstream link of PI3K with the MAP kinase pathway.

1.7.8 Phospholipase A_2

Mammalian cells express two main isoforms of phospholipase A_2 (PLA_2), namely cytosolic PLA_2 ($cPLA_2$) (Sharp *et al.,* 1991) and secretory PLA_2 ($sPLA_2$) (Kramer *et al.,* 1989): it is the former, cytosolic isoform which is involved in the generation of arachidonic acid (AA) and PAF (Ramesha and Pickett, 1990; Suga *et al.,* 1990; Lin *et al.,* 1992) from various integral membrane phospholipids, including PC, phosphatidylethanolamine (PE), and phosphatidylinositol (PI) (Berridge, 1985; Berridge, 1982). The activation of $cPLA_2$ is dependent upon both phosphorylation by MAPK-dependent and -independent pathways, and Ca^{2+}-dependent translocation to the plasma membrane (Fouda *et al.,* 1995; Lin *et al.,* 1992). The $sPLA_2$ is released extracellularly where it may play a role in membrane homeostasis, through its non-selective cleavage of *sn*-2 fatty acyl groups of outer-leaflet membrane phospholipids (Schalkwijk *et al.,* 1990; Dennis, 1983).

Neutrophil priming agents, including LPS (Fouda *et al.,* 1995; Doerfler *et al.,* 1994), GM-CSF (Durstin *et al.,* 1994; Gomez-Cambronero *et al.,* 1993), and TNFα (Bass *et al.,* 1994), have been shown to increase the mobilization and activity of both isoforms of PLA_2 and elicit a small, but significant release of AA. It has also been demonsrated that TNFα can increase the activity of co-enzyme A-independent transacylase (CoAIT) which maintains the phospholipid substrate levels for PLA_2 (Winkler *et al.,* 1994). Thus, it has been proposed that the enhanced availability of AA may contribute to the priming of subsequent responses by these agents (Durstin and Sha'afi, 1996; McDonald *et al.,* 1993). However, this idea has been disputed by

the demonstration that TNFα-induced priming of superoxide anion release occurs independently of AA release (Ely *et al.*, 1994).

Although AA may function as an intracellular mediator in its own right, it also forms the main precursor for the biosynthesis of eicosanoids (prostaglandins, leukotrienes and thromboxanes, figure 1.6). LPS has been reported to prime LTB_4 release in response to PMA, opsonised zymosan and calcium ionophore, but not to fMLP (Doerfler *et al.*, 1989). However, the priming of fMLP-stimulated superoxide anion release by TNFα and GM-CSF is unaffected by the inhibition of LTB_4 synthesis (Stewart *et al.*, 1991); thus, although the contribution of PLA_2 to lipid mediator release is unequivocal, its role in neutrophil priming is doubtful.

Various priming agents, including LPS (Stewart and Harris, 1991; Worthen *et al.*, 1988), TNFα (Stewart *et al.*, 1991), GM-CSF (De Nichilo *et al.*, 1991; Wirthmueller *et al.*, 1989) and PAF itself (Doebber and Wu, 1987), can increase PAF synthesis (and its release) in response to fMLP. It should be noted however that the majority of newly-synthesized PAF is not released but is retained within the neutrophil (Lynch and Henson, 1986). It has been suggested that PAF may act as an additional intracellular messenger for regulating $[Ca^{2+}]_i$ levels in human neutrophils during phagocytosis (Tool *et al.*, 1989), and that it may also contribute to the priming effects of LPS (Worthen *et al.*, 1988). However, since it has been demonstrated that the priming of fMLP-stimulated superoxide anion release by TNFα and GM-CSF is not affected by PAF receptor blockade (using WEB 2086, a specific antagonist of both the extracellular and the putative intracellular PAF receptor) (Stewart *et al.*, 1991), a role for intracellular PAF in neutrophil priming remains to be established.

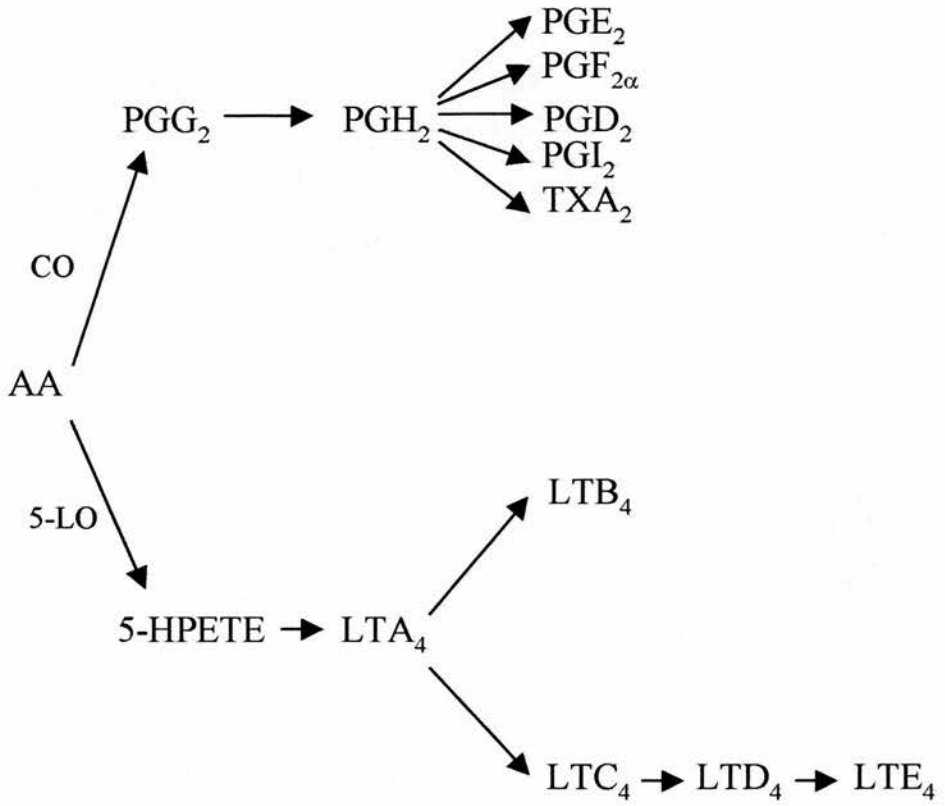


Figure 1.6
Arachidonic Acid Metabolism through Cyclo-oxygenase and 5-Lipoxygenase Pathways.
AA = arachidonic acid, CO = cyclo-oxygenase, PG = prostaglandin, TX = thromboxane, 5-LO = 5-lipoxygenase, 5-HPETE = 5-hydroperoxyeicosatetraenoic acid, LT = leukotriene.

1.7.9 Tyrosine Phosphorylation

Protein phosphorylation is an important mechanism of intracellular signalling in virtually all cells. Although the majority of phosphorylation reactions involve serine and threonine residues, a critical role for tyrosine phosphorylation also exists in the regulation of a diverse array of cellular functions, including neutrophil activation. Neutrophils express receptors with intrinsic tyrosine kinase activity (e.g. for growth factors) as well as numerous cytosolic tyrosine kinases (e.g. members of the *src* and *syk* family) (Taniguchi *et al.,* 1993; Akimaru *et al.,* 1992; Bolen *et al.,* 1992) that mediate the effects of agents such as LPS (English *et al.,* 1993) and opsonised particles (Unkeless *et al.,* 1992). Neutrophils also contain various tyrosine phosphatases that are responsible for the removal of phosphate residues (Tsui *et al.,* 1993; Fialkow *et al.,* 1993). In view of the central role of tyrosine phosphorylation in regulating a large number of neutrophil functions, including migration, NADPH oxidase activation, degranulation and phagocytosis (Fuortes *et al.,* 1993; Unkeless *et al.,* 1992), there has been considerable interest in characterizing the precise repertoire of tyrosine-phosphorylated proteins within the neutrophil and determining their exact functional roles.

The majority of neutrophil stimulants, including fMLP, PAF, TNFα, G-CSF, GM-CSF, PMA and substance P, have been shown to induce the tyrosine phosphorylation of a number of intracellular proteins (McColl *et al.,* 1991; Gomez-Cambronero *et al.,* 1989; Ohta *et al.,* 1992; Lloyds *et al.,* 1995; Akimaru *et al.,* 1992; Gomez-Cambronero *et al.,* 1991; Nick *et al.,* 1997; Rollet *et al.,* 1994). The concentration-dependency and time-course of tyrosine phosphorylation elicited by these agents correlates closely with their capacity to induce priming, thereby suggesting a mechanistic role. Furthermore, a marked similarity has been observed between the pattern of tyrosine phosphorylation produced by different priming agents, with proteins of 39-43 kDa, 72-76 kDa and 112-120 kDa consistently being detected (Hallett and Lloyds, 1995; Richard *et al.,* 1994). The demonstration that tyrosine phosphatase inhibitors (by augmenting tyrosine phosphorylation) can enhance both

respiratory burst activity and CD11b/CD18 expression of human neutrophils, whilst tyrosine kinase inhibitors prevent these responses (Naccache *et al.,* 1994; Lloyds and Hallett, 1994), further implicates a role for tyrosine phosphorylation in neutrophil priming.

The precise tyrosine kinases and target proteins involved in these events have not yet been identified. However, the demonstration that ligation and spatial clustering of neutrophil adhesion molecules (e.g. L-selectin and β_2-integrins) is linked to both enhancement of respiratory burst activity and the tyrosine phosphorylation and activation of p42/44 MAPK (Waddell *et al.,* 1995; Waddell *et al.,* 1994; Berton *et al.,* 1994), suggests a possible identity for the 39-43 kDa protein mentioned above (Hallett and Lloyds, 1995). Tyrosine phosphorylation of MAPK has also been reported following neutrophil priming with LPS (Fouda *et al.,* 1995) and GM-CSF (Gomez-Cambronero *et al.,* 1992; Okuda *et al.,* 1992), although not with TNFα (Waterman and Sha'afi, 1995). The MAPK cascade is a series of serine/threonine kinases whose activity depends upon phosphorylation of both tyrosine and threonine residues (Anderson *et al.,* 1990). Since both MAPK and a 72-kDa protein kinase (which may represent the 72-76 kDa protein mentioned above) have been shown to serine phosphorylate the terminal peptide sequence of the $p47^{phox}$ component of NADPH oxidase (Grinstein *et al.,* 1993), this may implicate their involvement in the downstream signalling events of certain priming agents. Indeed, it has been proposed that the tyrosine phosphorylation of both these proteins is required for Ca^{2+}-mobilizing agonists to fully activate $p47^{phox}$ (Hallett and Lloyds, 1995) and hence the respiratory burst. However, as with many of the signalling events that result from priming agonist exposure, the task of dissecting which events are critical to priming and which are linked to other neutrophil functions is formidable.

1.8 Neutrophil De-priming

The high rate of constitutive apoptosis observed in cultured neutrophils *in vitro* and the short circulating half-life of these cells *in vivo*, explains in part why most *in vitro* studies have focused on the short-term effects of priming agents and the cellular mechanisms responsible for them. The few studies that have examined the long-term priming effects of pro-inflammatory agents, such as LPS and G-CSF, have shown that enhanced respiratory burst capacity can be maintained for at least 24 hours, both in cultured human and sheep peripheral blood neutrophils (Carey *et al.,* 1994; Ichinose *et al.,* 1990).

A similar, sustained priming effect has been demonstrated using agitated suspension neutrophils, although these studies have been restricted to much shorter incubation periods. For example, it has been shown that priming of fMLP-stimulated superoxide anion release by LPS is maintained for at least 2 hours (Guthrie *et al.,* 1984), whereas GM-CSF and IFN-γ can augment the intracellular respiratory burst capacity of neutrophils for at least 4 hours (Roberts *et al.,* 1993). In view of these findings, it has been suggested that a sustianed neutrophil priming effect might be important *in vivo*, as part of the long-term inflammatory response observed with certain agents, including endotoxin (Carey *et al.,* 1994). Thus, neutrophil priming has been viewed as a permanent process, whereby neutrophils are maintained in a functionally up-regulated state for the duration of their life-span, in order to allow optimal resolution of widespread or prolonged inflammatory insults.

However, it has also been shown that intravascular *priming* of neutrophils can increase the magnitude of vascular injury induced by immune complexes and various chemotactic factors (Worthen *et al.,* 1987; Warren *et al.,* 1989). In fact, neutrophil priming appears to be a prerequisite for neutrophil-mediated host tissue damage (Smedly *et al.,* 1986). Hence, a more flexible regulation of neutrophil priming would appear to be in the host's best interest, in keeping with the fine tuning of other

neutrophil responses. Thus, if neutrophils primed either within the circulation or at the endothelial surface could revert *in situ* to an unprimed quiescent state, then this might represent a means of host tissue protection in the midst of an acute inflammatory response.

Despite the potential patho-physiological, and hence therapeutic, importance of being able to rescue neutrophils from the primed state, this area of research remains unexplored. This may reflect the practical difficulties encountered during neutrophil isolation, when endogenous priming by LPS yields a population of basally-primed neutrophils (Haslett *et al.,* 1985). Even if a pure population of totally *un-primed* neutrophils could ever be obtained *ex-vivo*, the ubiquitous nature of environmental LPS and the short neutrophil life-span will always confound subsequent *in vitro* investigations. Hence, very few studies have addressed the issue of whether neutrophil priming is reversible.

The few published demonstrations of apparently "reversible" priming have used physico-chemical stimuli to manipulate the primed responses of neutrophils. For example, hypotonic shock (Edashige *et al.,* 1993) and cell swelling (Miyahara *et al.,* 1993) have been reported to induce a temporary state of neutrophil priming that is abolished upon the restoration of isotonicity and cell size, respectively. However, these stimuli do not represent a physiological or ideal model of reversible priming, not least because they also induce secondary non-specific effects upon plasma membrane structures (resulting in the disorganization of intracellular microfilaments and an increase in cell-surface net negative charge (Miyahara *et al.,* 1993)). It is unlikely therefore that the reversible priming effects of physico-chemical stimuli are mediated through cell-surface receptors or give genuine insight into the potential for this to occur following physiological challenge. Hence, these data should be interpreted primarily as *in vitro* demonstrations that neutrophils can *de-prime* rather than evidence of any such potential effect *in vivo*. However, if a receptor-mediated biological agent that was part of the *in vivo* acute inflammatory response was shown to evoke a range of reversible functional responses *in vitro*, including the *gold*

standard of priming for superoxide anion generation, then this would represent a far more substantial finding.

1.9 Aims

The research for this thesis was concerned primarily with the potential for human neutrophils to revert from a primed to a quiescent state: this concept was referred to as *de-priming*.

(1) Preliminary investigations were aimed at determining optimal priming conditions for the selected agents, $InsP_6$, PAF and TNFα, and the establishment of a protracted (2 hour) *in vitro* incubation protocol that did not result in any loss in cell viability or priming potential.

(2) Several indices of priming were chosen, in order to encompass the range of neutrophil functions and ensure that neutrophil de-priming represented a global reversal of neutrophil responses rather than the specific desensitization of individual pathways.

(3) The potential for de-primed neutrophils to be re-primed, either with a homologous or a heterologous agonist, was determined as an evaluation of the true *reversibility* of neutrophil priming.

(4) A study of the potential mechanisms of neutrophil de-priming was undertaken, which included the manipulation of inherent priming responses with specific receptor antagonists.

2. CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

All reagents, unless otherwise stated in the text, were purchased from Sigma Chemical Company (Poole, Dorset, UK) and stored according to the manufacturer's instructions. Dextran-500 (MW 500,000) was dissolved (6% w/v) in sterile 0.9% saline and stored at 4 °C. PAF was dissolved (10 mM) in analar ethanol and stored at -80 °C. LPS (E. Coli serotype 0111:B4, γ-irradiated) was dissolved (1 mg/ml) in PBS, sonicated (ultrawave sonic bath, Belmont Instruments, Glasgow, UK) and stored at -20 °C. fMLP was dissolved (70 mg/ml) in DMSO (dimethyl sulfoxide), made up to 1 mM in PBS, and stored at -20 °C. PMA was dissolved (1 mg/ml) in DMSO and stored at -20 °C. Superoxide dismutase was dissolved (7500 U/ml) in PBS and stored at -20 °C.

Human recombinant TNFα was purchased (as a 1 ml solution in PBS) from Genzyme (Cambridge, MA) and stored at -80 °C: immediately prior to use, it was diluted in PBS according to the individual batch activity. Mouse monoclonal antibodies (mAb) were from various sources: anti-CD11b (44) and anti-CD11c (3.9) mAbs were from Serotec (Oxford, UK); anti-L-selectin mAb (Leu-8) was from Beckton Dickinson (Oxford, UK); anti-CD11a mAb (WAC 70) was donated by Dr. J. Ross (Dept. of Surgery, Edinburgh University); anti-TNF-R55, anti-TNF-R75, and anti-IL-2-R α-chain mAbs were from R & D Systems (UK); anti-phosphotyrosine (4G10) mAb was from Upstate Biotechnology Inc. (New York). FITC (fluorescein isothiocyanate)-conjugated $F(ab)_2$ fragments of rabbit anti-mouse immunoglobulin were from Dako (Buckinghamshire, UK), and peroxidase-conjugated, rabbit polyclonal, anti-mouse secondary antibody was from Amersham (UK). Optimal antibody binding concentrations were determined by titration.

2.2 The Isolation of Human Neutrophils from Peripheral Blood

The activation status of neutrophils can be profoundly affected during their isolation from peripheral blood (Norgauer *et al.,* 1991; Fearon and Collins, 1983; Haslett *et al.,* 1985). Both the isolation procedure itself and contamination with trace amounts of LPS can prime neutrophils, confounding their subsequent *in vitro* responses. It was therefore critical to select a method that yielded a pure population of minimally primed neutrophils *ex vivo*. Two preparative methods were investigated, and the subsequent functional responses of the isolated neutrophils were compared. Both methods yielded neutrophils with >99.5% viability (assessed by trypan blue exclusion) and >95% purity (assessed by examination of May-Grunwald-Giemsa-stained cytocentrifuge preparations) (Figure 2.1), with the <5% contamination consisting of <0.5% monocytes, 1-2% erythrocytes and 3-5% eosinophils. If >5% eosinophils were present the cells were discarded.

2.2.1 The Isolation of Human Neutrophils using Discontinuous Plasma-Percoll Gradients

Human neutrophils were isolated exactly as previously detailed (Haslett *et al.,* 1985), using sterile, LPS-free (<0.1 ng/ml LPS by the Chromagenix *Limulus* amoebocyte lysate assay) reagents and plastic-ware (Falcon, Oxford, UK). This method has been shown to yield neutrophils that have minimal alterations in both their priming status (with respect to fMLP-induced superoxide anion generation) (Haslett *et al.,* 1985) and their resting cell morphology (<8% shape change assessed by flow-cytometry) (Cole *et al.,* 1995). All procedures were carried out at room temperature, unless otherwise stated.

Figure 2.1
Representative Cytospin Preparation of Freshly-Isolated Human Neutrophils.
100 µl of freshly isolated human neutrophils (10^7 cells/ml in PBS with $CaCl_2$ and $MgCl_2$) were spun onto a glass slide (300*g*, 3 min, cytocentrifuge) and stained with May-Grunwald-Giemsa (×400).

Peripheral venous blood was collected from healthy adult volunteers, into 50 ml polyethylene tubes containing 4 ml of the anticoagulant sodium citrate (3.8%, Phoenix Pharmaceuticals Ltd., Gloucester, UK), to a total volume of 40 ml. The blood tubes were then centrifuged (300*g*) for 20 min. Each supernatant of platelet-rich plasma was carefully aspirated and either centrifuged (2500*g*, 20 min) to produce platelet-poor plasma (PPP), or used to prepare autologous serum at 37 °C, by the addition of 20 μM $CaCl_2$. 5 ml dextran-500 (6%) was added to the remaining cell pellets, and 0.9 % saline was added to give a final volume of 50 ml. The contents of each tube were mixed gently and thoroughly, and left for 30-40 min to allow erythrocyte sedimentation to occur.

The overlying, leukocyte-rich, plasma layer was aspirated and centrifuged (300*g*, 6 min), and the mixed leukocyte cell pellet was resuspended in 2 ml PPP and transferred to a 15 ml polystyrene tube. The leukocytes from two 50 ml tubes of blood could be used for each gradient. A stock solution of 90 % v/v Percoll was prepared in 0.9% saline, and was used to prepare fresh density gradients of 42% and 51% Percoll in PPP. The accurate preparation of the 51% Percoll step was critical for the complete separation of neutrophils from erythrocytes. The resuspended cell pellet was then underlayered sequentially with 2 ml of each of the 42% and 51% Percoll gradients, and centrifuged (275*g*, 10 min). Polymorphonuclear cells were harvested from a wide band at the interface of the 42% and 51% Percoll layers. Mononuclear cells remained in a thinner band at the top, PPP/42% Percoll, interface and erythrocytes pelleted at the bottom of the tube. The purified neutrophils were washed sequentially in PPP (500*g*, 6 min), PBS without, and PBS with $CaCl_2$ and $MgCl_2$ (endotoxin-free, pH 7.4, 300*g*, 6 min). Cell concentrations were adjusted according to haemocytometer counts.

2.2.2 The Isolation of Human Neutrophils using Discontinuous PBS-Percoll Gradients

A second neutrophil isolation procedure, using PBS-Percoll gradients (Dooley *et al.*, 1982), was compared with the plasma-Percoll method. The initial part of the separation procedures were identical, but diverged following aspiration of the leukocyte-rich plasma layer. An isotonic stock solution of 90% v/v Percoll was made with 10× PBS. The mixed leukocyte cell pellet was resuspended in 2.5 ml 55% Percoll in PBS, and transferred to a 15 ml polystyrene tube. Density gradients of 70% and 81% Percoll in PBS were prepared, and 2.5 ml of each was used to sequentially underlayer the resuspended cell pellet. The gradients were centrifuged (700*g*, 20 min), and polymorphonuclear cells were collected from the 70%/81% Percoll interface. Mononuclear cells remained at the top 55%/70% Percoll interface, and erythrocytes pelleted at the bottom of the tube. The purified neutrophils were washed sequentially in PBS without plus PPP, PBS without, and PBS with $CaCl_2$ and $MgCl_2$ (300*g*, 6 min). Cell concentrations were adjusted as above, according to haemocytometer counts.

2.2.3 The Comparison of Neutrophil Isolation Procedures

The activation status of neutrophils isolated by the two outlined methods was compared, using parallel assays of respiratory burst activity (cytochrome C reduction, lucigenin-dependent chemiluminescence, dihydrorhodamine 123 oxidation), shape change, and adhesion molecule expression (see below for Methods). There was no significant difference between neutrophils isolated by the two different methods (Figure 2.2), in: (i) the resting cell morphology; (ii) the basal expression of L-selectin or CD11b; (iii) the basal or fMLP-induced intracellular respiratory burst activity (measured by the oxidation of dihydrorhodamine 123). However, superoxide anion release (measured by the reduction of cytochrome C or lucigenin-dependent chemiluminescence) appeared to be a much more sensitive indicator of neutrophil activation status. A significant increase in fMLP-stimulated superoxide anion release, the "gold standard" indicator of neutrophil priming, was

observed with neutrophils isolated by the PBS-Percoll method (Figure 2.3). Since this indicated that a small degree of endogenous neutrophil priming had occurred during this isolation procedure, the plasma-Percoll method was selected for all further investigations.

2.3 The Assessment of Respiratory Burst Activity in Human Neutrophils

Neutrophil priming was originally defined as an enhancement of respiratory burst activity. It can be quantified by measuring several different parameters of NADPH oxidase activity: (i) an increase in oxygen consumption; (ii) superoxide anion generation; (iii) hydrogen peroxide production; and (iv) the overall, intracellular respiratory burst activity. Of these, the enhancement of fMLP-stimulated superoxide anion release has been viewed as the gold standard indicator of neutrophil priming. The generation of superoxide anions is the initial step of the neutrophil respiratory burst, where oxygen is converted to reactive oxygen species. During phagocytosis, the bulk of these anions is released from neutrophils into the extracellular milieu. Therefore, the measurement of extracellular superoxide anion release correlates directly with the neutrophil's total microbicidal potential. Furthermore, it can be measured by simple and quantitative assays.

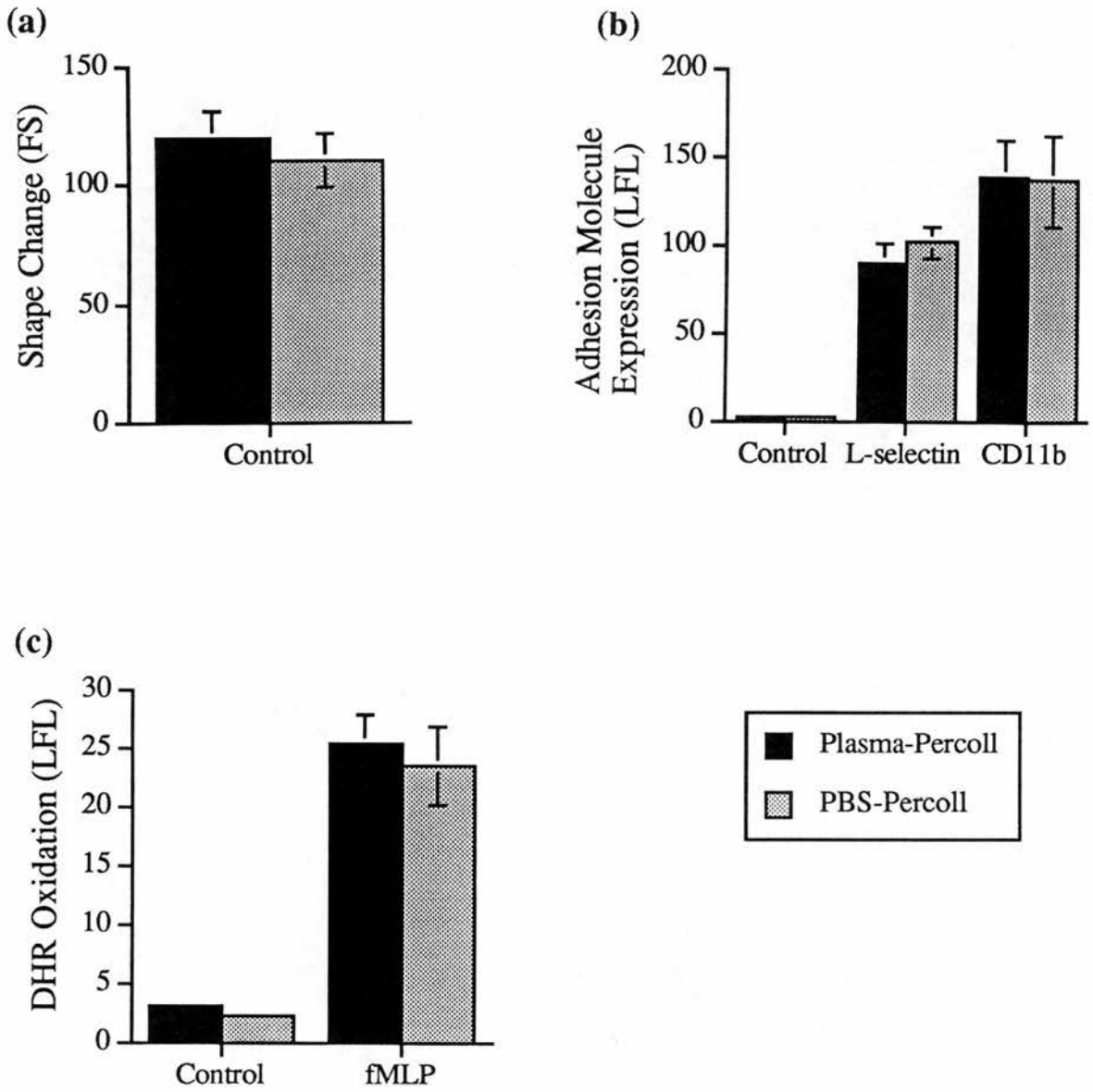


Figure 2.2

Comparison of Freshly-Isolated Human Neutrophils Prepared by Discontinuous Plasma-Percoll or PBS-Percoll Gradients.

Neutrophils (from the same donor) were isolated in parallel by either plasma-Percoll or PBS-Percoll gradients and resuspended in PBS (10^7 cells/ml). Parallel assays of: (a) shape change (EPICS flow cytometry, see 2.4.2); (b) expression of L-selectin and CD11b (see 2.5.1); and (c) intracellular respiratory burst activity (DHR oxidation, see 2.3.4) were then performed on neutrophils incubated with 100 nM fMLP or buffer control.

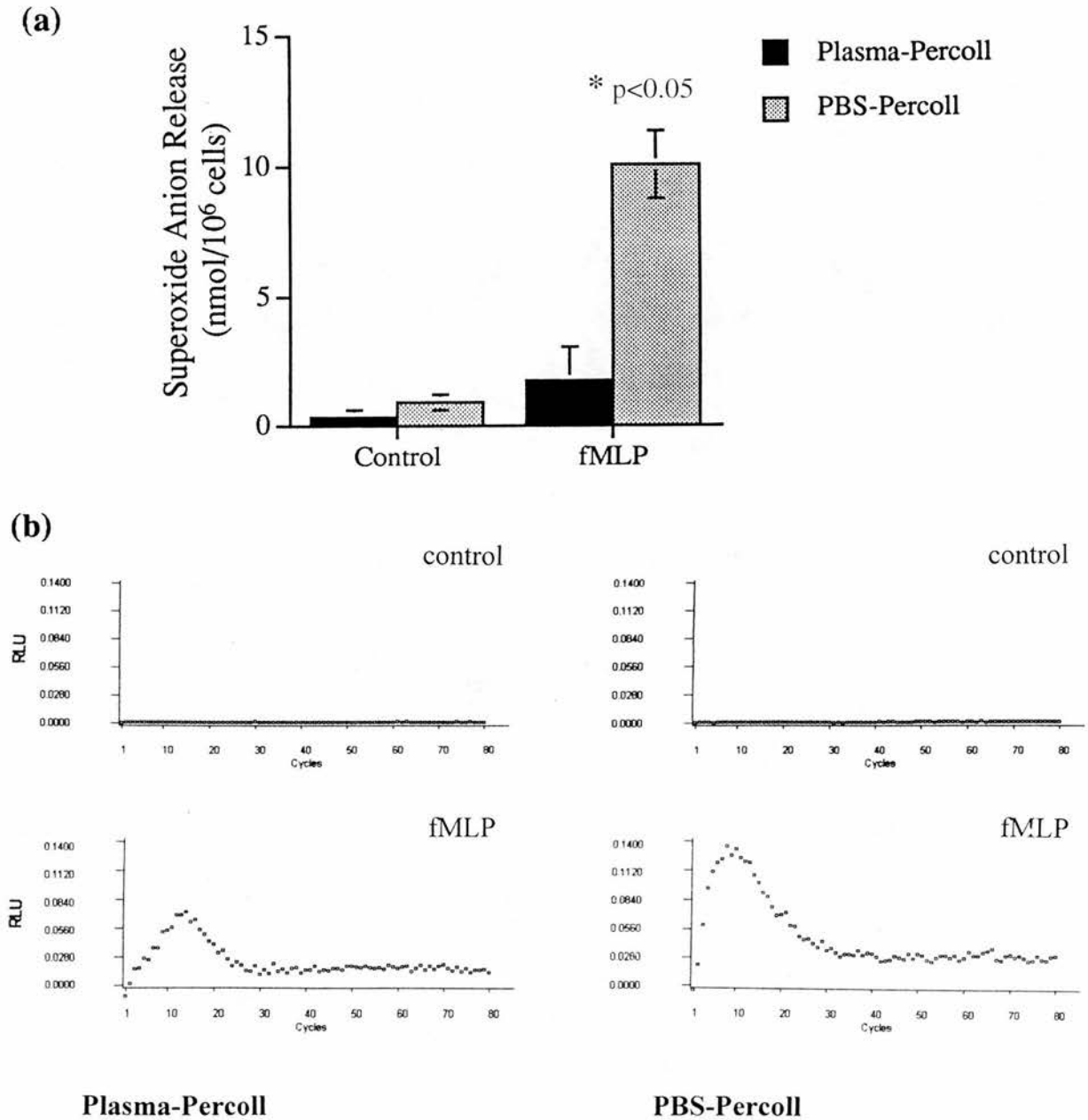


Figure 2.3

Comparison of Superoxide Responses of Freshly-Isolated Human Neutrophils Prepared by Discontinuous Plasma-Percoll or PBS-Percoll Gradients.

Neutrophils (from the same donors as fig. 2.2) were isolated in parallel by either plasma-Percoll or PBS-Percoll gradients and resuspended in PBS (10^7 cells/ml). Superoxide anion responses of buffer-treated control neutrophils and neutrophils stimulated with 100 nM fMLP were then assessed in parallel assays of: (a) cytochrome C reduction (see 2.3.1, values represent mean ± SEM for triplicate determinations from the 3 donors in figure 2.2); and (b) lucigenin-dependent chemiluminescence (see 2.3.2, histograms from a single donor representative of 3).

2.3.1 The Measurement of Superoxide Anion Generation using Cytochrome C

Cytochrome C is an electron-accepting compound that can be reduced by superoxide anions, giving an increase in absorbance at 550 nm which can be detected with a spectrophotometer. However, cytochrome C reduction is not specific for superoxide anions, therefore superoxide dismutase (a superoxide-specific enzyme) must be included in parallel samples to reveal the amount of residual oxidation by other respiratory burst products. The superoxide dismutase-inhibitable reduction of cytochrome C is a very sensitive and well established assay of cumulative superoxide anion release from neutrophils (Haslett *et al.,* 1985; Guthrie *et al.,* 1984; Babior *et al.,* 1973).

Neutrophils, isolated by the plasma-Percoll method above, were resuspended immediately in PBS with $CaCl_2$ and $MgCl_2$. Aliquots of 1×10^6 neutrophils (70-90 µl) were transferred to 2 ml sterile, polypropylene Eppendorf tubes, and allowed to equilibrate at 37°C, upon gentle shaking (110 cycles/min) in a water-bath (Haake) for approximately 5 min. Neutrophil priming was initiated by adding a 10 µl aliquot of the appropriate priming agent or buffer control and incubating the neutrophils for the specified time period. A volume of 800 µl pre-warmed cytochrome C (horse heart preparation, final concentration 1 mg/ml in PBS with $CaCl_2$ and $MgCl_2$) was then added, followed immediately by 100 µl fMLP (final concentration 100nM), PMA (final concentration 100 ng/ml) or buffer control, to give a final incubation volume of 1.0 ml. One of each set of quadruplicate samples included 375 U superoxide dismutase. Incubations were continued in the shaking water-bath for a further 10 min (fMLP) or 60 min (PMA), whereupon samples were placed on ice to terminate the reaction.

The cells were removed by centrifugation (12,000*g*, 2 min, 4°C) and supernatants were transferred to 1.5 ml spectrophotometer cuvettes with a standard light path of 1 cm. The optical density of each supernatant was quantified at a wavelength of 550 nm (OD_{550}) using a Pye-Unicam 8700 spectrophotometer that scanned between 535-

565 nM, with the baseline provided by 1 mg/ml cytochrome C. The reduction of cytochrome C was determined for each sample, using the extinction coefficient ΔE_{550} = 21.0 × 10^3/M/cm (Massey, 1959), for the absorption of reduced minus oxidized cytochrome C. For a sample volume of 1 ml and a light path of 1 cm, the observed OD_{550} was multiplied by 47.6, as 1 mol superoxide anions reduces 1 mol cytochrome C: upon subtraction of corresponding superoxide dismutase-inhibited samples, this yielded the nmols superoxide anions generated per 10^6 neutrophils.

2.3.2 The Measurement of Superoxide Anion Generation using Lucigenin-Dependent Chemiluminescence (LDCL)

Chemiluminescence (CL) allows kinetic analysis of the respiratory burst activity of neutrophils. Oxygen radicals that are produced during the respiratory burst can react with, and thus excite, biological substrates, which then relax to their ground state by photon emission. This energy release is in the form of light, which can be amplified by chemiluminescent probes (Allen and Loose, 1976) and measured in a luminometer. Lucigenin is such a chemiluminescent probe, being an acridinium salt which reacts specifically with superoxide anions (Williams and Cole, 1981) to produce electronically-excited N-methylacridone. Lucigenin-dependent CL (LDCL) therefore provides a rapid and convenient assay of the kinetics of superoxide anion generation, whilst, at the same time, allowing a quantification of the cumulative superoxide anion response that can be compared with that obtained by cytochrome C reduction.

Freshly prepared lucigenin (bis-*N*-methylacridinium nitrate, 0.25 mM in 100 µl PBS containing 1 mg/ml BSA) was added to individual wells of white polystyrene microtitre plates (Dynatech Laboratories, Billinghurst, U.K.) and allowed to thermally equilibrate for approximately 30 min at 37°C. It has previously been shown that both the rate of onset and the peak height of the neutrophil CL response is dependent upon the cell concentration (Blair *et al.*, 1988), with 1 × 10^6 neutrophils per 250 µl well (of a 96-well plate) being sufficient for rapid kinetic analysis of

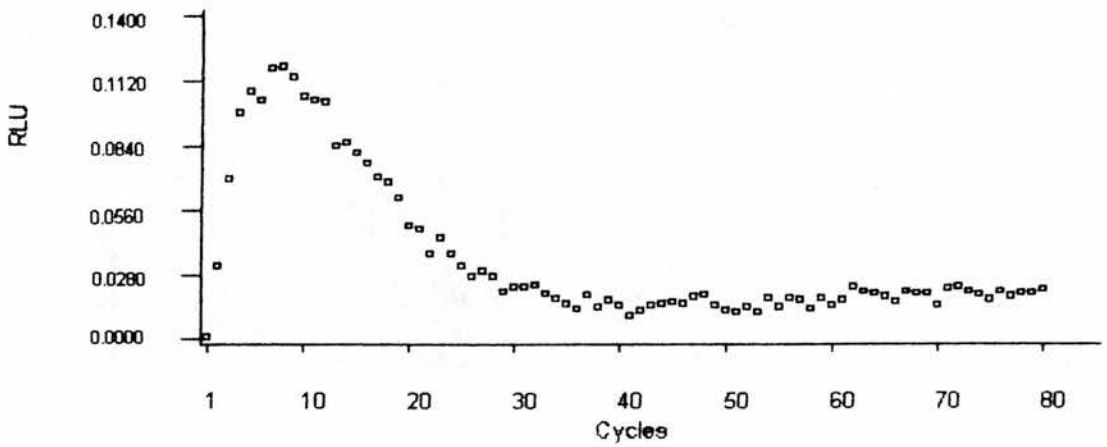
superoxide anion release. Therefore, 1×10^6 neutrophils (70 µl in PBS with $CaCl_2$ and $MgCl_2$) were transferred into 2 ml sterile Eppendorf tubes and equilibrated at 37°C in a shaking water-bath. Quadruplicate samples were incubated for the required time-period with a 10 µl volume of priming agent or buffer control.

Neutrophils were then transferred into the pre-warmed, lucigenin-containing microtitre plates, with control wells of lucigenin alone or lucigenin plus neutrophils (made up to 180 µl with PBS). Neutrophils were immediately treated with 20 µl fMLP (final concentration 100 nM) or buffer, and loaded into a shaking, ML 3000 luminometer (Dynatech Laboratories Ltd., Billinghurst, UK) pre-heated to 37°C. Chemiluminescence activity (relative light units, RLU) was recorded at 17 s intervals, and subsequent analysis (Cellular Chemiluminescence, Dynatech Laboratories Ltd.) allowed the peak height and cumulative CL activity (area under the curve) to be determined from individual wells (Figure 2.4a).

2.3.3 The Correlation of Chemiluminescence Values: Peak Height versus Area Under Curve

Previous reports have used several, different parameters to describe the CL response, including (i) the maximum rate of increase in CL, (ii) the time to peak, (iii) the peak height, and (ii) the integrated area under the curve. Since the area under the curve provides a measure of the cumulative CL activity whilst encompassing both the rate of increase in CL and the peak height, it is considered the most useful parameter for inter-assay comparisons (Blair *et al.*, 1988). However, the CL responses of neutrophils treated under different conditions have been shown to differ markedly, ranging from a rapid, well-defined peak (e.g. with fMLP (Bender and Van Epps, 1983; Stocks *et al.*, 1995)) to a sustained plateau (e.g. with PMA (Schult *et al.*, 1985)). Therefore, we compared the peak height and integrated area values for neutrophils treated under a variety of assay conditions (Figure 2.4b): a direct correlation ($y = 40.24x + 0.78$, $r^2 = 0.78$) was found between these two parameters, in agreement with previous findings (Van Dyke and Van Dyke, 1986).

(a)



(b)

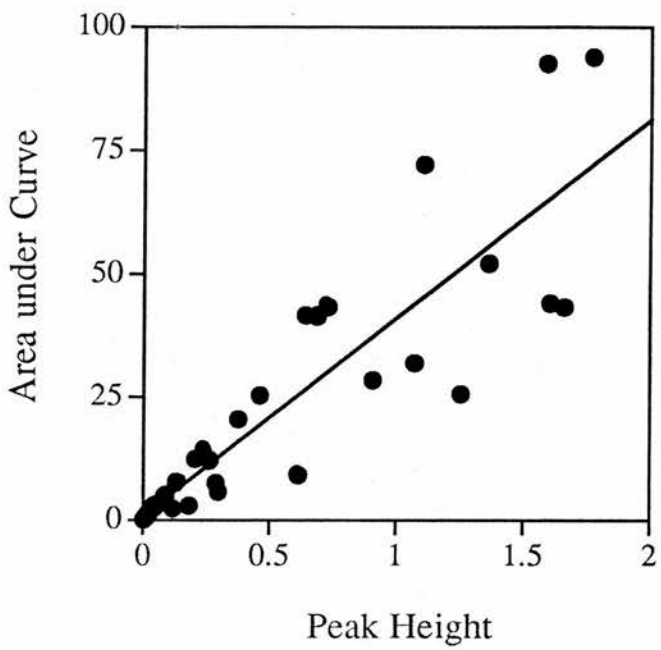


Figure 2.4

Assessment of Superoxide Anion Generation by Lucigenin-dependent Chemiluminescence.

Neutrophils were incubated with PAF (1 μM, 10 min) or buffer, prior to treatment with fMLP (100 nM) or buffer in the presence of lucigenin (25 mM plus 1 mg/ml BSA). Kinetic chemiluminescence activity (relative light units, RLU) was then assessed. (a) Representative chemiluminescence data for neutrophils treated with 100 nM fMLP. (b) Correlation of peak height and area under curve values for cumulative data.

2.3.4 The Measurement of Intracellular Respiratory Burst Activity using Dihydrorhodamine 123 (DHR)

The chemiluminescence and cytochrome C reduction assays both provide a measure of the cumulative respiratory burst activity of the total neutrophil population. However, these assays give no quantitative information about the actual responses of individual cells. Since neutrophils are not a homogeneous cell population, but exist as functionally distinct sub-populations (Daniels *et al.,* 1994; Klempner and Gallin, 1978), one cannot assume that all cells are behaving identically. Even within a homogeneous sub-population, a sub-optimal concentration of a stimulus could cause a graded response, with partial activation of all cells, or cause an "all-or-nothing" response, with activation of some cells and no effect on others (Daniels *et al.,* 1994; Bass *et al.,* 1983). Dihydrorhodamine 123 (DHR) is a non-fluorescent, membrane-permeable, cytometric probe (Kinsey *et al.,* 1987), that is taken up by phagocytes, and oxidized during the respiratory burst into the green-fluorescent, rhodamine 123 (Emmendorffer *et al.,* 1990). It has thus been used as a sensitive measure of intracellular respiratory burst activity (Rothe *et al.,* 1991; Royall and Ischiropoulos, 1993; Emmendorffer *et al.,* 1990). Flow-cytometric analysis allows the quantification of respiratory burst activity at the single cell level, as well as the percentage of neutrophils within each subpopulation.

Since DHR can be rapidly oxidized in air, preliminary experiments assessed the intra-assay stability of DHR. Neutrophils (1×10^6 in PBS with $CaCl_2$ and $MgCl_2$) were transferred in duplicate 90 µl aliquots to sterile 2 ml Eppendorf tubes, and allowed to thermally equilibrate in a shaking water-bath at 37°C. A stock solution of DHR (final assay concentration 1 µM) was freshly prepared in PBS, and warmed to 37 °C. A 700 µl volume of PBS with either 50 µl DHR or buffer control was then added to the neutrophils, followed immediately by 10 µl priming agent or buffer for the appropriate time period. A 50 µl aliquot of DHR was then added to those samples that had been incubating with buffer alone, with the same volume of buffer added to those already containing DHR. Neutrophils were incubated with 100 µl

fMLP (final concentration 100 nM) or buffer for 10 min, whereupon the reaction was stopped by placing the cells on ice.

The cell-associated green fluorescence of 5000 neutrophils per sample was analyzed immediately, using an EPICS Profile II flow cytometer (Coulter Electronics, Luton, UK). The log fluorescence intensity (LFL) was plotted against neutrophil number, allowing the percentage of neutrophils within each sub-population to be determined (Figure 2.5). However, in the vast majority of samples, a single neutrophil population was observed and, therefore, the mean LFL was taken as a direct measurement of DHR oxidation and, hence, of the mean respiratory burst activity per neutrophil.

Preliminary investigations demonstrated a direct relationship between the duration of the DHR incubation with neutrophils and the LFL value (Figure 2.6). Thus, in order to standardize all further studies, DHR was added immediately prior to the priming incubation.

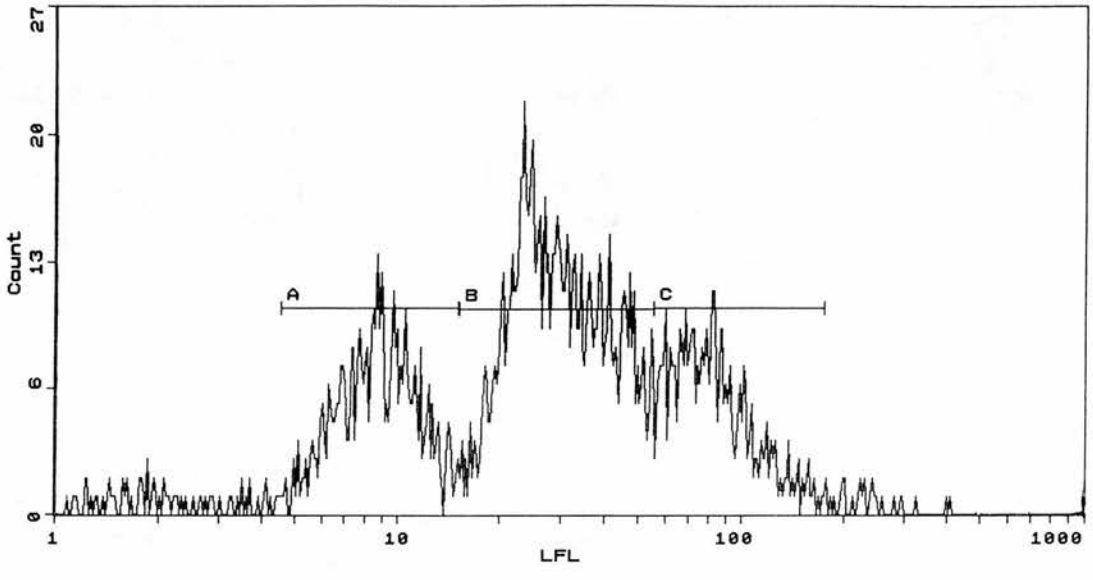


Figure 2.5
Assessment of Intracellular Respiratory Burst Activity by Dihydrorhodamine 123.

Representative flow-cytometry (EPICS Profile II) histogram showing distinct neutrophil sub-populations (gated as A, B and C) following treatment with TNFα (200 U/ml, 30 min) then fMLP (50 nM, 10 min), in the presence of 1 μM DHR (x-axis shows logarithmic scale green fluorescence (LFL) and y-axis shows relative cell number).

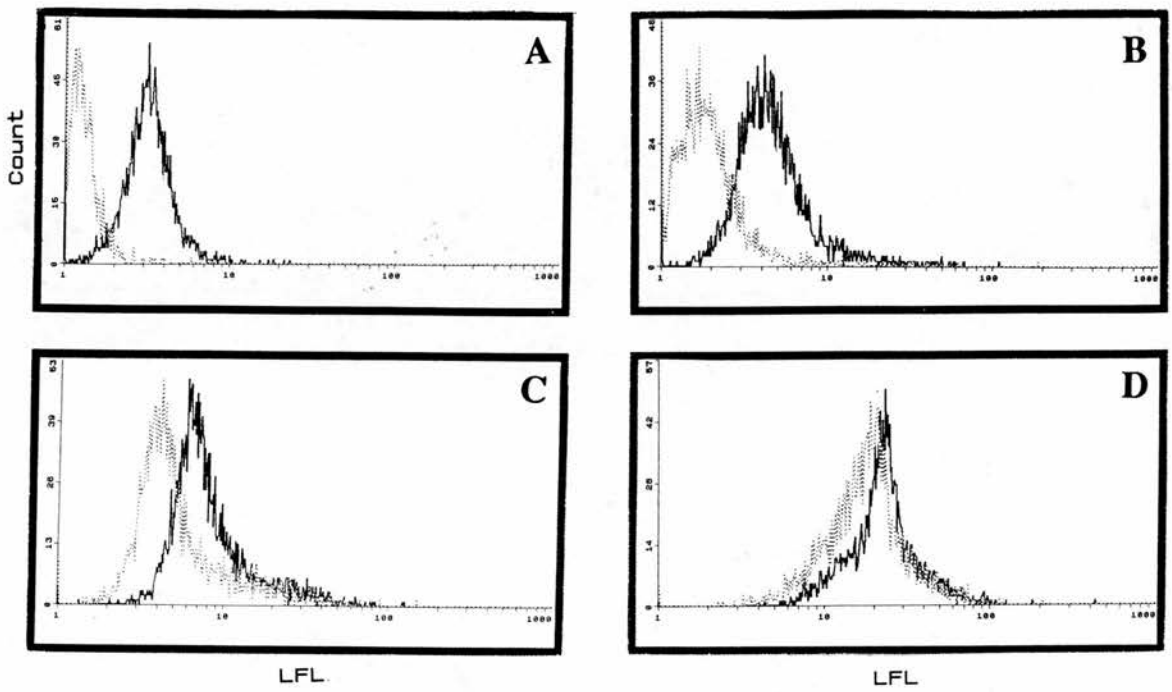


Figure 2.6
Dependence of Log Fluorescence Values on the Dihydrorhodamine 123 Incubation Time with Human Neutrophils.
Neutrophils were incubated with PBS alone or PBS containing DHR (final concentration 1 μM, black outlines), followed immediately by buffer (A and C) or PAF (1 μM, B and D) for 10 min. DHR was then added to the remaining samples (light grey outlines) prior to a 10 min treatment with buffer (A and B) or 100 nM fMLP (C and D). Samples were analyzed by flow cytometry (x-axis shows logarithmic scale green fluorescence (LFL) and y-axis shows relative cell number). Representative flow-cytometry (EPICS Profile II) histograms (n = 3 in triplicate).

2.3.5 The Correlation Between Assays of Neutrophil Respiratory Burst Activity

Although LDCL, cytochrome C reduction, and DHR oxidation have been used to measure respiratory burst activity, the inter-relationships between these assays have not been established. Thus, parallel experiments were performed with human neutrophils under a variety of incubation conditions (10^6 neutrophils/ml treated with PAF (1 μM, 10 min), TNFα (200 U/ml, 30 min) or appropriate buffer controls, followed by incubation with fMLP (100 nM, 10 min), PMA (100 ng/ml, 60 min), or buffer), and respiratory burst activity was assessed by the three different methods detailed above. Although a direct, linear relationship ($y = 0.57x + 0.34$, $r^2 = 0.62$) was shown between the LDCL (area under curve) and cytochrome C reduction methods of assessment, which have both been used to specifically measure superoxide anion generation (Haslett *et al.,* 1985; Babior *et al.,* 1973; Williams and Cole, 1981), this association was relatively weak (Figure 2.7a) and did not appear to depend on the stimulus used (individual data not shown). Since the cytochrome C reduction assay was able to detect lower levels of neutrophil priming than LDCL, it was used for all further experiments.

A direct, non-linear, correlation ($y = 0.57x^{1.40}$) was demonstrated between the measurement of cytochrome C reduction and the oxidation of DHR (Figure 2.7b), as reported previously for opsonized zymosan-stimulated neutrophils (Smith and Weidemann, 1993). Since DHR is a measure of intracellular oxidant production, including H_2O_2 (Royall and Ischiropoulos, 1993; Rothe *et al.,* 1991), it was used alongside cytochrome C reduction as a complementary measure of respiratory burst activity.

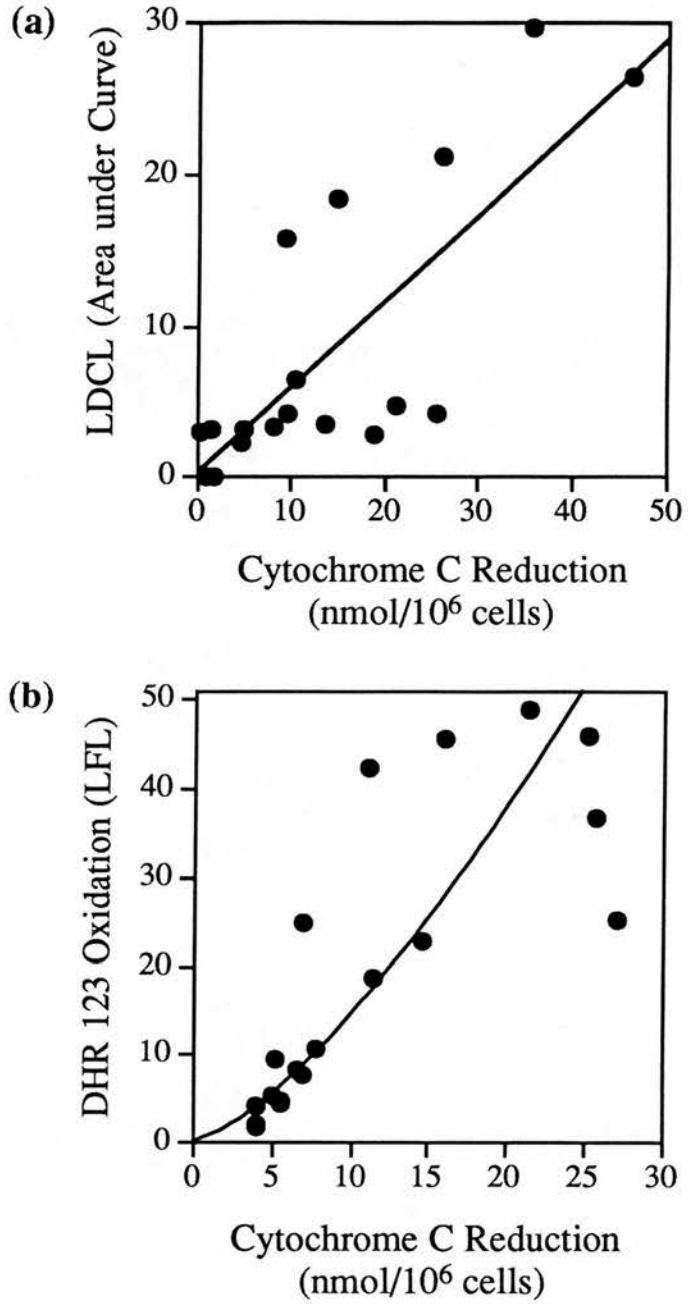


Figure 2.7
Correlation of Assays for Neutrophil Respiratory Burst Activity.
(a) Correlation between chemiluminescence (area under curve) and cytochrome C reduction values for parallel samples. (b) Correlation between DHR oxidation (log fluorescence, LFL) and cytochrome C reduction values for parallel samples.

2.4 The Assessment of Neutrophil Shape Change

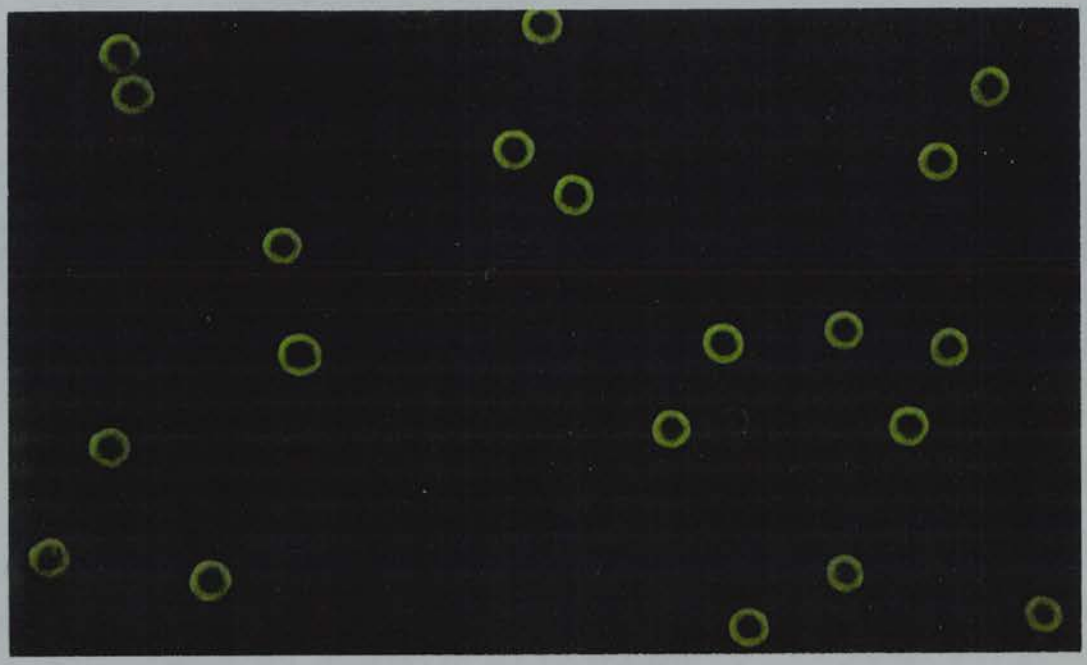
Although the enhancement of respiratory burst activity has been viewed as the gold standard indicator of neutrophil priming, it may be accompanied by changes in other important neutrophil responses. Thus, a biological mediator may act as a priming agent, having no effect alone, but being able to enhance a subsequent response to a secretagogue agonist (e.g. respiratory burst activity), whilst simultaneously acting to directly activate other responses (e.g. shape change, aggregation, adhesion molecule up-regulation). One of the earliest responses that can be observed when a neutrophil is primed is the extrusion of pseudopodia from an otherwise spherical cell: this shape change has been shown to be a very sensitive indicator of priming (Haslett *et al.*, 1985).

Freshly-isolated neutrophils (1×10^6 in 90 µl PBS with $CaCl_2$ and $MgCl_2$) were allowed to equilibrate in 2 ml Eppendorf tubes, in a shaking water-bath at 37°C. Agonists or corresponding buffer controls were added in a 10 µl volume to duplicate samples, and incubated with neutrophils for the appropriate time-periods. Neutrophils were subsequently fixed by the addition of 1 ml glutaraldehyde (final concentration 2.5% in PBS) and assessed by either light microscopy or flow cytometry.

2.4.1 The Light-Microscopic Evaluation of Neutrophil Shape Change

Microscopic quantification of shape change has been shown to correlate with chemotaxis measured using Boyden chambers (Haston and Shields, 1985). Glutaraldehyde-fixed neutrophils were assessed visually by phase-contrast light microscopy (Figure 2.8). Shape change was defined as the percentage of neutrophils that extruded more than one pseudopodia, and was calculated for a minimum of 300 cells per sample (established by a stable running mean).

A



B

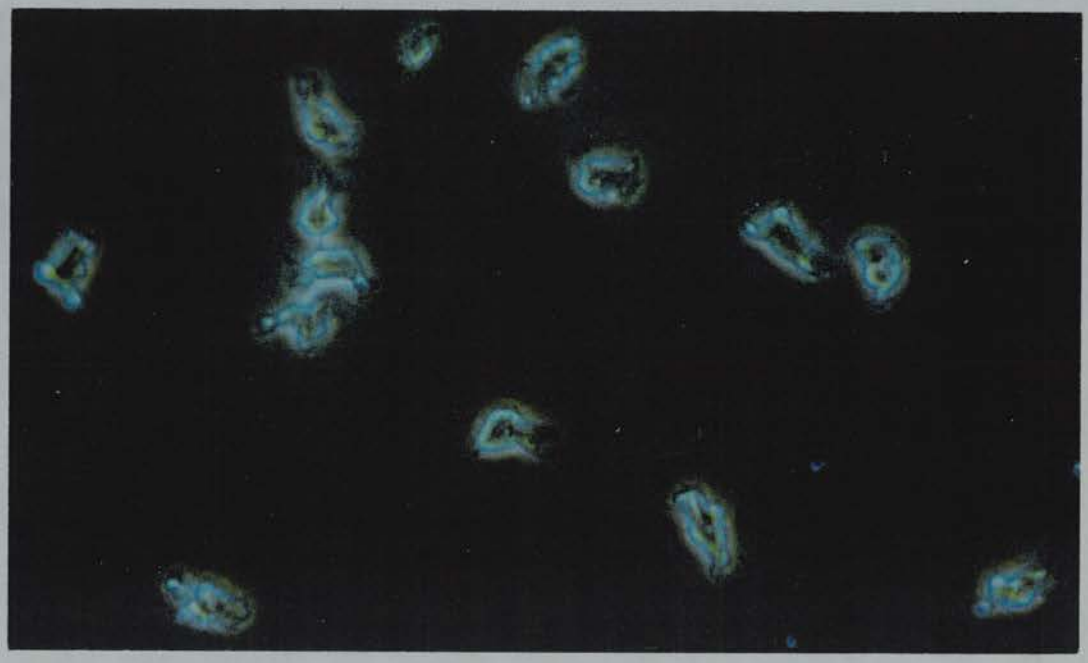


Figure 2.8
Assessment of Neutrophil Shape Change by Polarizing Light-Microscopy.
Representative photographs of neutrophils incubated for 10 min with (A) buffer or (B) 100 nM fMLP, as seen by polarizing light microscopy (courtesy of Dr. Jiamin Qu, Babraham Institute, Cambridge).

2.4.2 The Flow-Cytometric Evaluation of Neutrophil Shape Change

Neutrophils that are defined as shape-changed by light microscopy have been shown to cause a greater scatter of oncoming light than spherical cells (Keller *et al.*, 1995; Cole *et al.*, 1995). This increased forward light scatter is the basis of a rapid and objective assay of shape change, which avoids the potential observer bias of light microscopy.

The mean forward light scatter (FS) of 5000 cells per sample was determined using an EPICS Profile II flow cytometer. The non-shape changed (spherical) neutrophil population was determined from control samples and was gated out as previously detailed (Cole *et al.*, 1995), allowing the remaining, shape-changed neutrophils to be quantified as a percentage of the total neutrophil population (Figure 2.9a).

2.4.3 The Correlation Between Assays of Shape Change

The values obtained by flow-cytometric assessment of neutrophil morphology (% shape change) showed a direct, but non-linear, correlation ($y = 6.79x^{0.58}$) with those derived by light-microscopic evaluation (Figure 2.9b). However, the flow-cytometric method tended to slightly overestimate (by approximately 6%) the extent of basal shape change, and slightly underestimate (by 3%) the maximal shape change, when compared with the microscopic assessment of cell morphology. These findings are similar to a previous report, where flow-cytometric quantification of the shape change induced by various chemotactic factors tended to overestimate basal shape change by 8%, and underestimate maximal shape change by 5% (Cole *et al.*, 1995). Despite these small differences of quantification at the two extremes of neutrophil shape change, the flow-cytometric method of analysis provided a more convenient and objective measure of shape change than light microscopy, and was thus preferred when large numbers of samples were generated.

2.5 The Assessment of Neutrophil Adhesion Molecule Expression and Activation

Neutrophil priming agents can regulate the expression of a variety of plasma membrane glycoproteins, including the leukocyte adhesion molecules CD11b/CD18 and L-selectin (Borregaard *et al.,* 1994). However, CD11b/CD18 can exist in an inactive form at the cell surface, therefore its expression may not necessarily correlate with its functional activity. Indeed, priming agents can differentially regulate the expression and function of neutrophil adhesion molecules (Condliffe *et al.,* 1996). Therefore, both the expression of neutrophil adhesion molecules and the functional capacity of CD11b/CD18 were taken as further indices of priming.

2.5.1 The Analysis of Adhesion Molecule Expression by Flow Cytometry

The surface expression of neutrophil adhesion molecules was quantified by indirect immunofluorescence, as previously detailed (Dransfield *et al.,* 1992). Purified neutrophils (4×10^6 in 900 µl PBS with $CaCl_2$ and $MgCl_2$ plus 25 mM HEPES) were incubated at 37 °C in 2 ml Eppendorf tubes, with 100 µl priming agent or buffer controls for the specified time period. The reactions were stopped by placing the cells on ice.

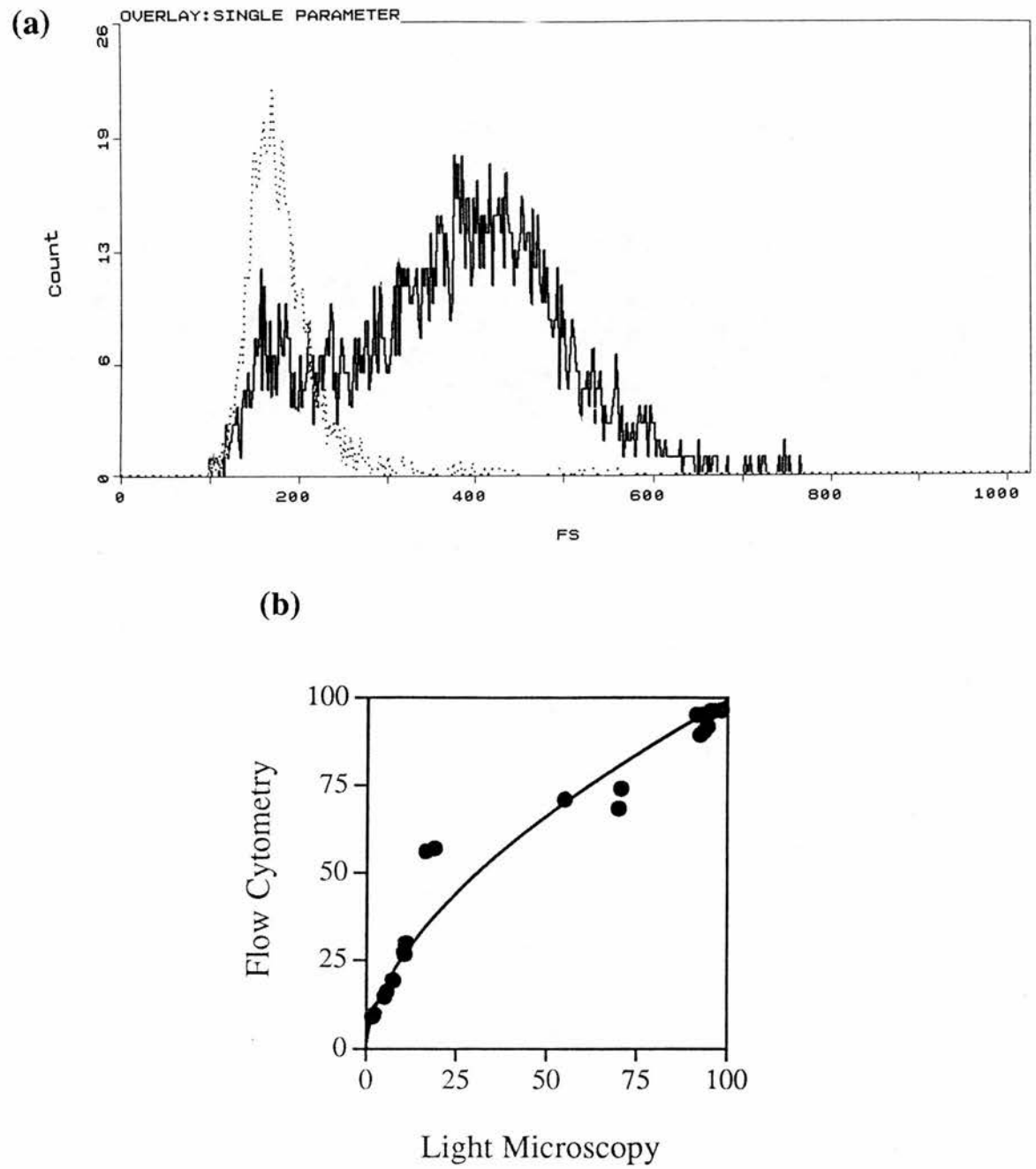


Figure 2.9
Assessment of Neutrophil Shape Change by Flow-Cytometry.
(a) Representative flow-cytometry (EPICS Profile II) histogram showing neutrophils incubated with TNFα (50 U/ml, 30 min, black outline) and control neutrophils (light grey outline) (x-axis shows mean forward light scatter (FS) and y-axis shows relative cell number). Percent shape change was calculated by gating out the population of non-shape changed neutrophils determined from control samples. (b) Correlation between the light-microscopic and flow-cytometric evaluation of percentage shape change for parallel samples.

A 50 µl aliquot of these neutrophils (2×10^5) was resuspended in 25 µl saturating concentrations of the specified monoclonal antibody, in respective wells of a 96-flexiwell plate, and incubated for 30 min (4 °C). The neutrophils were subsequently washed three times (1000*g*, 60 s, 4 °C) in PBS containing 0.2 % BSA and 0.1 % sodium azide, prior to a further 30 min incubation (4°C) with 25 µl FITC-conjugated, goat, anti-mouse immunoglobulin (diluted 1:25 in PBS/BSA/azide), and washed again. Neutrophils (5000 cells per sample) were analyzed immediately using an EPICS Profile II flow cytometer, with fluorescence values recorded on a logarithmic scale (LFL). The mean LFL value was used as a direct measurement of adhesion molecule expression.

2.5.2 The Analysis of CD11b Activation by Albumin-Coated Latex Bead (ACLB) Binding

The functional capacity of plasma-membrane CD11b/CD18 was determined by flow cytometry, upon the binding of fluorescent, albumin-coated latex beads (ACLB) (Stocks *et al.,* 1995). Fluorescent latex beads (2.5% v/v stock solution, Polysciences Inc, Warrington, PA) were washed three times in PBS, resuspended (2.5% v/v) in human serum albumin (10 mg/ml in PBS) for 10 min at 25°C, then washed (3×) prior to resuspension (0.75% v/v) in PBS.

Aliquots of freshly isolated neutrophils (1.75×10^6 in 175 µl PBS with $CaCl_2$ and $MgCl_2$) were thermally equilibrated in sterile 2 ml Eppendorf tubes in a shaking water-bath at 37°C, then incubated with 25 µl priming agent or buffer control for the specified time period. Fifteen minutes before the termination of each reaction, a 25 µl aliquot of ACLB (0.75% v/v solution) was added to each tube; for time-points less than 15 min, the beads were added before the agonist. Reactions were stopped by the addition of 0.5 ml glutaraldehyde fixative (0.5%), then neutrophils were subsequently left for 30 min at 25 °C. Non-adherent ACLB were removed by three washes in PBS.

The log green fluorescence (LFL) of neutrophils (5000 cells per sample) was determined using an EPICS Profile II flow cytometer. Since the LFL value is directly proportional to the number of ACLB bound, distinct neutrophil sub-populations could be distinguished with increasing numbers of ACLB: the number of neutrophils with one or more attached ACLB was calculated as a percentage of the total neutrophil population, by gating out the control population (with no attached ACLB) (Figure 2.10).

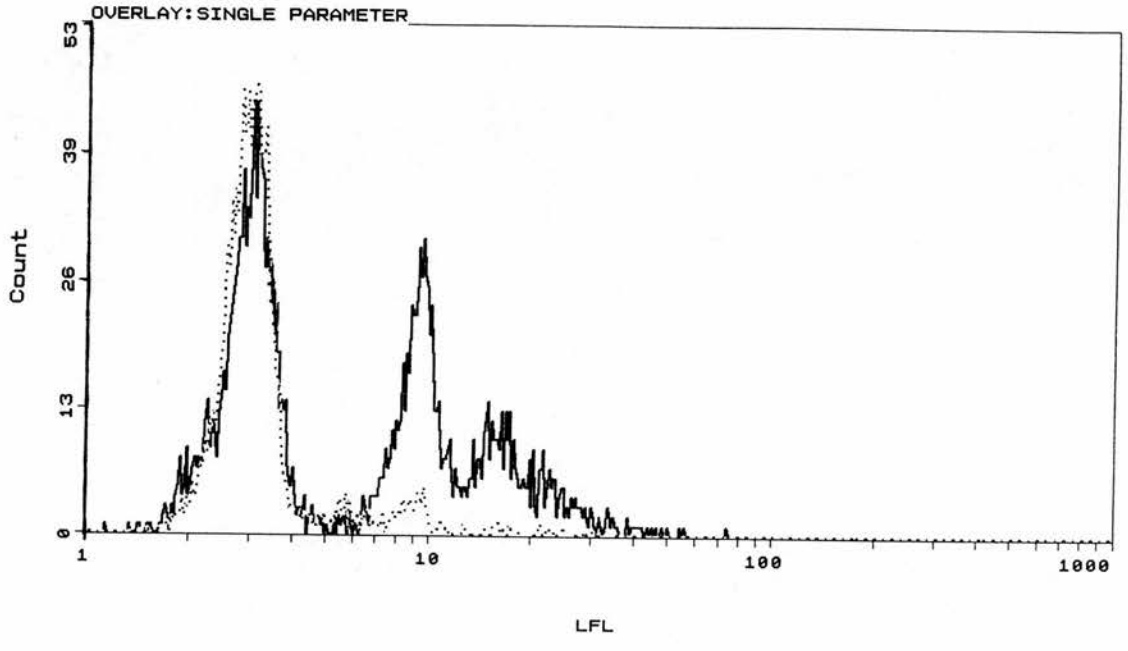


Figure 2.10
Assessment of CD11b Activation by Albumin-coated Latex Bead Binding.
Representative flow-cytometry (EPICS Profile II) histogram showing ACLB binding to neutrophils following treatment with PAF (1 μM, 10 min, black outline) or buffer control (light grey outline) (x-axis shows logarithmic scale green fluorescence (LFL) and y-axis shows relative cell number). The percentage of neutrophils with attached ACLB was calculated by gating out the far left peak determined from control samples.

2.6 Immunoblotting of Phosphorylated Tyrosine Residues in Human Neutrophils

2.6.1 Extraction of Proteins from Human Neutrophils using TCA

Freshly-isolated neutrophils were resuspended in PBS with $CaCl_2$ and $MgCl_2$. Aliquots of 4×10^6 neutrophils (360 µl) were transferred to 2 ml sterile, polypropylene Eppendorf tubes, and allowed to equilibrate at 37°C, upon gentle shaking (110 cycles/min) in a water-bath (Haake, Sweden) for approximately 5 min. Neutrophils were then treated with a 40 µl aliquot of PAF (1 µM, 10 min or 120 min), TNFα (100 U/ml, 30 min or 120 min) or buffer control (10 min, 30 min or 120 min). Reactions were stopped at the appropriate times by the addition of 1.6 ml ice-cold HEPES (20 mM)-buffered saline containing protease inhibitors (50 µg/ml leupeptin, 20 µg/ml aprotinin, and 1 mM AEBSF) (HBSI), and neutrophils were collected by centrifugation (7,500*g*, 1 min, 4 °C).

Cell pellets were then immediately resuspended in 0.5 ml HBSI plus 0.5ml 20% TCA, vortexed, and incubated at 4 °C for 10 min. TCA extracts were sedimented (10,000*g*, 10 min, 4 °C), and lipids were extracted by resuspension in 1 ml ice-cold ethanol. Following sedimentation (10,000*g*, 10 min, 4 °C), protein extracts were washed in 1 ml d.d. H_2O (10,000*g*, 10 min, 4 °C), taken up in 10 µl d.d. H_2O, resuspended in 190 µl boiling (2×)-Laemmli sample buffer (LSB: 0.125 M TRIS-HCl, 4% SDS, 20% glycerol, 2.5 mM dithiothreitol, 0.01% bromophenol blue, 50 µg/ml leupeptin, 20 µg/ml aprotinin, and 1 mM AEBSF, pH 6.8) and boiled for 5 min in a heated water-bath (Grant, UK). Cooled samples were then incubated with 10 mM iodoacetamide for 20 min (25 °C), centrifuged (14,000g, 5 min, 25 °C), and supernatants were stored at 4 °C prior to analysis.

2.6.2 Extraction of Proteins from Human Neutrophils using Laemmli Sample Buffer

As an alternative method of protein extraction, the cell pellets (obtained after termination of the priming incubation and centrifugation in HBSI) were taken up in 25 µl HBSI, immediately resuspended in 175 µl boiling LSB, vortexed, and boiled for 20 min. Following centrifugation (14,000*g*, 5 min, 25 °C), supernatants were again stored at 4 °C.

2.6.3 Separation of Protein Extracts by Polyacrylamide Gel Electrophoresis (PAGE) and Electroblotting onto Nitrocellulose Membranes

20 µl of each sample and 20 µl pre-boiled (5 min, 100 °C) molecular weight markers (Rainbow markers, Biorad, CA) were loaded into individual lanes of a pre-cast 4-20% TRIS-glycine polyacrylamide gel (Biorad, CA). PAGE was then conducted (Mini Protean II apparatus, Biorad, CA) in Running Buffer (1.5 M TRIS base, 0.5% SDS, pH 8.8) at 30 mA constant current for approximately 2 hours. Parallel gels were either: (i) stained for total protein content with Coomassie Brilliant Blue (0.25% plus 10% glacial acetic acid) for 4 hours, followed by 4× 4 hour washes in 40% methanol/10% glacial acetic acid; or (ii) used for protein transfer onto nitrocellulose membranes (Hybond C, Amersham, UK) (400 mA, 4 hours), using Mini Protean II transfer apparatus, Biorad, CA) filled with Transfer Buffer (5 mM TRIS base, 5 mM glycine, 0.01% SDS).

Comparison of Coomassie-stained gels showed that TCA-extracted samples contained greater numbers of prominent protein bands than samples extracted by the boiling LSB method (Figure 2.11). Therefore, the TCA extraction protocol was selected for all further studies.

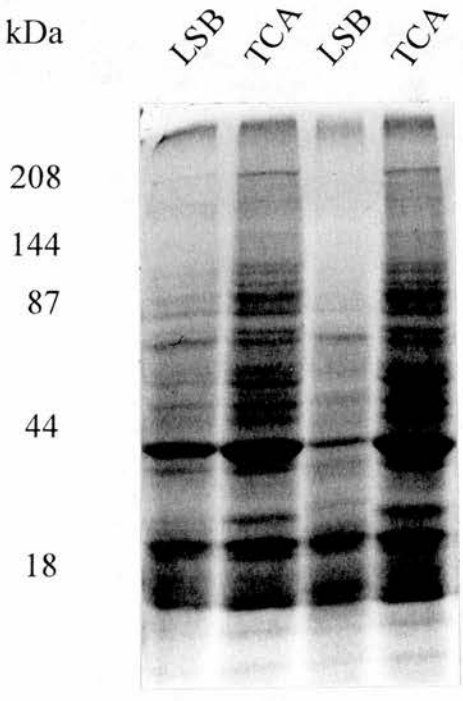


Figure 2.11
Comparison of TCA and SDS Methods of Neutrophil Protein Extraction.
Human neutrophils were incubated with buffer for 10 min. Reactions were stopped at the appropriate times with 20% TCA or boiling LSB (see text). Representative Coomassie Brilliant Blue-stained PAGE gel (n = 3). Biorad Rainbow markers.

2.6.4 Detection of Tyrosine Phosphorylated Proteins

Following protein transfer to nitrocellulose membranes, non-specific sites were blocked overnight in 50 ml Blocking Buffer (150 mM NaCl, 20 mM TRIS-HCl, 0.02% Tween-20, 5% powdered fat-free milk (Marvel), pH 7.4), on a rocking platform at 4 °C. Membranes were then incubated at 25 °C for 2 hours, in 50 ml Blocking Buffer containing mouse monoclonal, anti-phosphotyrosine antibody (4G10 1:5000, Upstate Biotechnology Inc., New York). This was followed by three sequential washes (30 min, 25 °C) in Blocking Buffer, incubation for 2 hours with a peroxidase-conjugated, rabbit polyclonal, anti-mouse, secondary antibody (1:5000, Amersham, UK) in Blocking Buffer, and a further three washes in Blocking Buffer alone. Immuno-labelled tyrosine-phosphorylated proteins were then detected by enhanced chemiluminescence (ECL, Amersham, UK) and exposure to photographic film (20 sec, Kodak XAR).

2.7 Statistical Analysis of Data

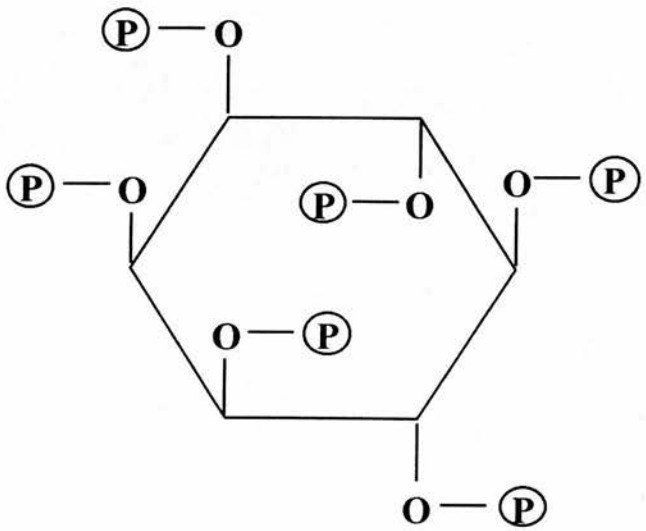
All values are expressed as means ± S.E.M. of (n) separate experiments. Values, where applicable, were compared by ANOVA or two-tailed Students' t-test for paired data, with $p<0.05$ considered to be significant. Significant differences between experimental conditions were determined by the Newman-Keuls procedure. Where relationships between different assays were found to be linear, the squares of the Pearson correlation coefficient (r) are given: for non-linear correlations, mathematical models were generated (Cricket Graph III, Computer Associates Inc., USA).

3. CHAPTER 3: CHARACTERIZATION OF THE FUNCTIONAL EFFECTS AND BINDING PROPERTIES OF INOSITOL HEXAKISPHOSPHATE IN HUMAN NEUTROPHILS

3.1 Introduction

Inositol hexakisphosphate ($InsP_6$) (Figure 3.1) is a ubiquitous, cytosolic molecule, which has been reported to have effects upon human neutrophils *in vitro* that are not seen with other inositol polyphosphates (Eggleton *et al.,* 1991). This study showed that $InsP_6$ (250 μM) had no effect on basal respiratory burst activity, but could prime the fMLP-induced superoxide anion response after an optimal 30 s pre-incubation period: this priming effect was transient, being diminished by approximately 40% after 5 min. In a subsequent study, $InsP_6$ (100 μM) was shown to increase the assembly of cytoskeletal F-actin after an optimal 5 min incubation period (Crawford and Eggleton, 1992), an effect which also underwent a significant reduction by 10 min.

Since $InsP_6$ is abundant in mammalian cells, being present at concentrations of up to 1 mM (Szwergold *et al.,* 1987), it was postulated that $InsP_6$ might play an important proinflammatory role *in vivo* upon its release from necrotic cells within an inflammatory focus (Eggleton *et al.,* 1991). This role as an extracellular mediator would require specific cell-surface receptors, through which $InsP_6$ could mediate its functional effects. Specific [^{3}H]-$InsP_6$ binding sites have been identified in membranes derived from various mammalian tissues, including: rat cerebellum (Hawkins *et al.,* 1990); rat heart (Rowley *et al.,* 1996); bovine adrenal chromaffin cells (Regunathan *et al.,* 1992); rat anterior pituitary and cerebral cortex (Nicoletti *et al.,* 1990). Furthermore, the $InsP_6$ receptor from rat cerebellum has been identified as the α-subunit of AP-2 (Voglmaier *et al.,* 1992), a molecule which has an important role in endocytosis.



$InsP_6$

Figure 3.1
The Molecular Structure of $InsP_6$.

The primary aim of the work in this chapter was to re-examine the transient nature of $InsP_6$-mediated priming effects in human neutrophils, in an attempt to initially confirm the findings of Eggleton and colleagues and, thereafter, establish a model with which to investigate the potential reversibility of neutrophil priming. The receptor-dependence of the $InsP_6$-mediated priming effect was also addressed.

3.2 Methods

3.2.1 The Buffering of $InsP_6$ Solutions

$InsP_6$ possesses six, negatively-charged, phosphate groups (figure 3.1), making it both a powerful Ca^{2+}-chelator (Cosgrove, 1980), and an acid when in solution ($InsP_6$ is also known as phytic acid). Therefore, it was essential to select a buffer that could prevent pH fluctuations *in vitro*. Of the 5 buffers tested (Figure 3.2), PBS with Ca^{2+} and Mg^{2+} plus 25 mM HEPES was chosen because: (i) it produced a stable physiological pH for $InsP_6$ concentrations $\leq$100 μM; (ii) it contains Ca^{2+} and Mg^{2+}, which are required for respiratory burst activation and shape change of neutrophils; and (iii) it is colourless and therefore does not interfere with the spectrophotometric cytochrome C reduction assay for superoxide anion generation.

3.2.2 The Priming of Neutrophils by Hypotonic Shock

To examine the priming effects of a hypotonic challenge, neutrophils (1×10^6 in 250 μl PBS with $CaCl_2$ and $MgCl_2$) were equilibrated at 37 °C and incubated for 19 min in 730 μl PBS containing cytochrome C (1 mg/ml), with either 150 mM NaCl (isotonic incubations) or 50 mM NaCl (hypotonic incubations). Neutrophils were then treated for 1 min with 20 μl of 5 M NaCl (to reverse hypotonicity to isotonicity) or PBS (to retain hypotonicity or isotonicity), prior to stimulation with 10 μl fMLP (final concentration 100 nM). One sample of each set of triplicates included 375 U superoxide dismutase. At the end of the incubation, samples were placed on ice, and the cells removed by centrifugation (12,000*g*, 2 min, 4 °C). Supernatants were then transferred to 1.5 ml spectrophotometer cuvettes and superoxide anion generation was calculated as detailed in the Methods Chapter (2.3.1).

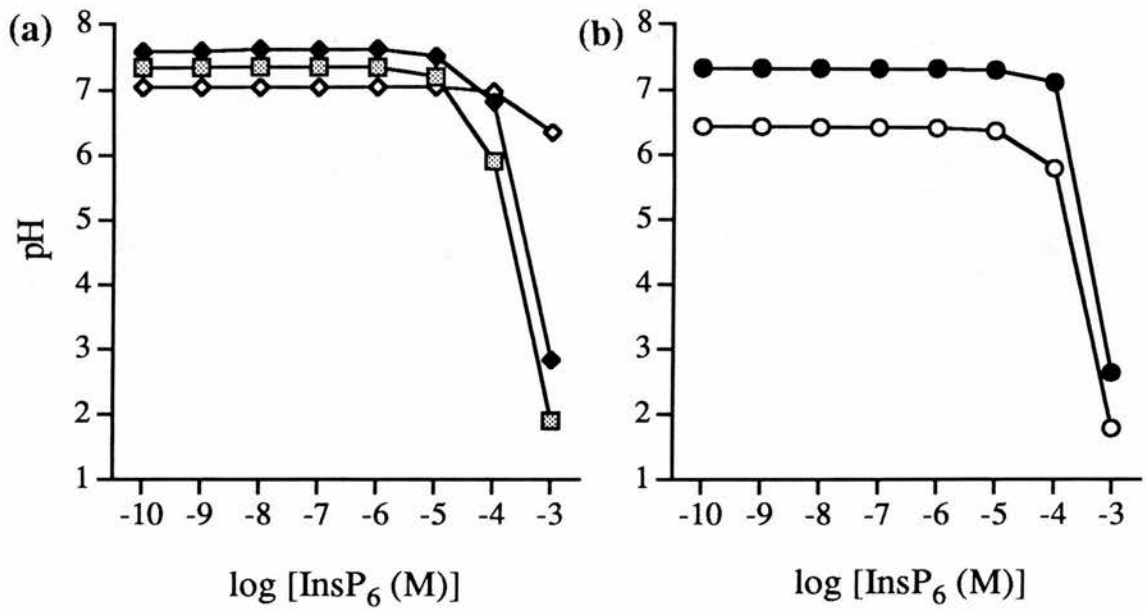


Figure 3.2
pH Profiles of Buffers containing $InsP_6$.
$InsP_6$ (0.1 nM-1 mM) was dissolved in the following buffers: (a) HBSS plus Ca^{2+} and Mg^{2+} (closed diamonds); NKET buffer (light squares); HBSS with Ca^{2+} and Mg^{2+} plus 25 mM HEPES (open diamonds); (b) PBS with Ca^{2+} and Mg^{2+} plus 25 mM HEPES (closed circles); PBS with Ca^{2+} and Mg^{2+} plus 25 mM MOPS (open circles). The pH of each solution was determined using an Orion pH meter (Boston, USA) (n = 3).

3.2.3 [^{3}H]$InsP_6$ Binding to Cell Membranes

[^{3}H]$InsP_6$ binding to human neutrophil and rat cerebellar membranes was performed as previously described (Hawkins *et al.*, 1990). All procedures were carried out at 4 °C, unless otherwise stated. Briefly, freshly isolated rat cerebella or human neutrophils (15×10^6/ml) were resuspended in 20 volumes of 20 mM NaCl, 100 mM KCl, 5 mM EDTA, 20 mM TRIS pH 7.7 (NKET buffer), and homogenized (3× 10s bursts in a Polytron homogenizer, set on maximum). The membrane suspensions were centrifuged (35,000*g*, 30 min) and the resulting membrane pellets washed twice in NKET buffer before use. Protein concentrations were determined for each pellet using the Pierce-BCA protein assay (with BSA as standard), and the membranes were finally resuspended at 0.1 mg protein/ml in NKET buffer.

Duplicate incubations, using freshly-prepared membranes, were performed with 0-10 nM [^{3}H]$InsP_6$ (specific activity 15-24 Ci/mmol, DuPont-New England Nuclear, Stevenage, UK) for the specified time period, in a final volume of 1 ml. Non-specific binding (NSB) was determined in parallel incubations, in the presence of 100 μM unlabelled $InsP_6$. In competition assays, displacing agents (unlabelled $InsP_6$, Ins(1,3,4,5,6)P_5 (Calbiochem, Nottingham, UK), and Ins(1,4,5)P_3 (Research Biochemicals International, St Albans, UK)) were added in 100 μl volumes.

Incubations were terminated by centrifugation (13,000*g*, 6 min, 4 °C) to separate bound from free radioligand, and membrane pellets were washed rapidly (×2) in NKET buffer. Pellets were dissolved overnight in Soluene, taken up in 1 ml scintillation fluid (Packard Fluoroscint 4, Pangbourne, UK) and analyzed for membrane-bound radioactivity by liquid scintillation counting (Packard Emulsafe, Pangbourne, UK). Values were expressed as a percentage of maximal specific [^{3}H]$InsP_6$ binding, where specific binding represented the total amount of membrane-associated [^{3}H]$InsP_6$ minus the non-specific binding.

3.2.4 [^{3}H]$InsP_6$ Binding to Intact Neutrophils

Two techniques were used to investigate [^{3}H]$InsP_6$ binding to intact human neutrophils. All procedures were carried out at 4 °C, unless otherwise stated.

The first method was a modification of the cell membrane binding protocol, suitable for use with intact cells. Freshly isolated neutrophils (3×10^6/ml, equivalent to 0.1 mg protein/ml) were incubated for 90 min with 0-10 nM [^{3}H]$InsP_6$, in a final volume of 1 ml of either 25 mM HEPES-buffered PBS (pH 7.5) or NKET buffer (pH 7.7). NSB was determined in parallel incubations containing 100 μM unlabelled $InsP_6$. Bound and free radioligand were separated by centrifugation (3000*g*, 2 min) followed by two washes in the appropriate buffer. Neutrophils were dissolved overnight in Soluene, and membrane-bound radioactivity was determined by liquid scintillation counting.

The second method included the use of an inert oil cushion to facilitate the separation of bound from free radioligand (O'Flaherty *et al.*, 1990). Neutrophils (3×10^6) were layered over 400 μl silicone oil (F-50, Croylek Ltd., Surrey, UK) in 2 ml Eppendorf tubes, incubated as detailed above for 90 min, and then centrifuged through the oil cushion (13,000*g*, 1 min). Parallel incubations were also performed using 10 pM [^{3}H]LTB_4 (approximately 45,000 dpm ± 100 nM unlabelled LTB_4 (NSB)) as a positive control, since intact neutrophils are known to bind LTB_4 (O'Flaherty *et al.*, 1990). The cell pellets and 200 μl samples of supernatants were isolated separately, dissolved in 0.5 ml methanol for 10 min, taken up in 2 ml scintillation fluid, and assessed for radioactivity by liquid scintillation counting.

3.2.5 Analysis of [^{3}H]$InsP_6$

In order to assess whether there was any metabolism of [^{3}H]$InsP_6$ during the 90 min incubation with neutrophil membranes, pre- and post-incubation supernatants were analyzed by anion-exchange H.P.L.C. (Hawkins *et al.*, 1990). A 5-SAX H.P.L.C. column (Partisphere 250 × 4.6 mm, Whatman Chromatography, Maidstone, UK)

fitted with a Whatman guard cartridge, was eluted at a flow rate of 1.25 ml/min with the following gradient: A (H_2O), B (3.5 M ammonium formate pH-adjusted to 3.7 with orthophosphoric acid): 0-5 min 0% B; 10-12 min 21.4% B; 18-23 min 28.5% B; 30 min 40.0% B; 40 min 42.0% B; 60-65 min 100% B. Fractions, collected every 18s, were mixed with 4 ml scintillation fluid, and measured for radioactivity by liquid scintillation counting.

3.3 Results

3.3.1 Functional Studies with $InsP_6$

3.3.1.1 Neutrophil Priming by Hypotonic Shock

Before pursuing the priming characteristics of $InsP_6$, we required verification of the ability of neutrophils to undergo transient priming in our system. The only previously documented protocol for inducing reversible priming of superoxide anion generation in human neutrophils was the use of hypotonic shock (Edashige *et al.*, 1993), and we therefore selected this model for re-examination. Using a modification of the published hypotonic challenge protocol, fMLP (100 nM) alone elicited little superoxide anion release under isotonic conditions, but this effect was primed (approximately 2-fold) by a previous 20 min hypotonic challenge (Figure 3.3). This modest, but significant, priming effect was completely reversed when isotonicity was restored 1 min prior to fMLP stimulation. Thus, in agreement with Edashige *et al.* (1993), we were also able to observe the phenomenon of reversible priming. We proceeded, therefore, to investigate the potential for neutrophils to de-prime under more physiological conditions (i.e. following receptor-mediated stimulation) using the putative biological priming agent $InsP_6$.

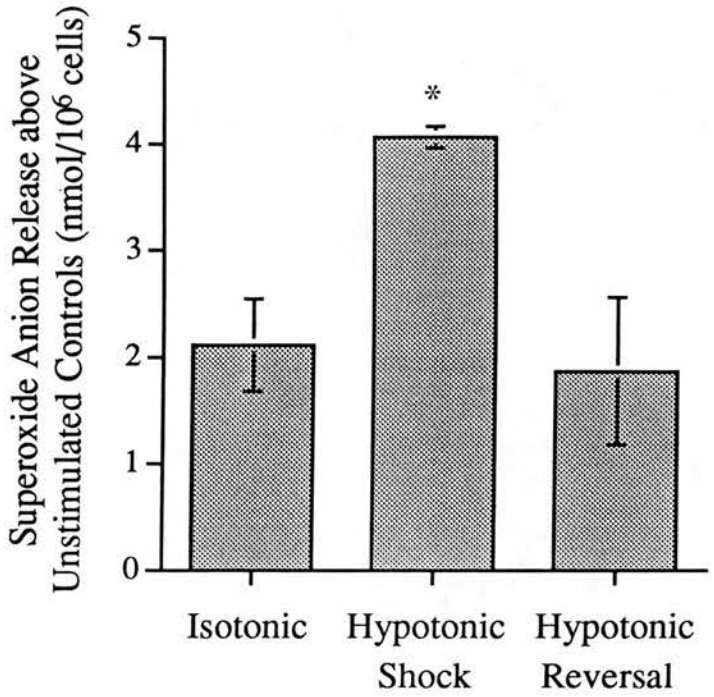


Figure 3.3
Priming of Human Neutrophils with a Hypotonic Challenge.
Neutrophils were incubated for 19 min in isotonic PBS (150 mM NaCl) or hypotonic PBS (50 mM NaCl), then treated for 1 min with 5M NaCl (hypotonic reversal) or PBS (to retain iso-or hypotonicity), prior to a 10 min stimulation with 100 nM fMLP or buffer (unstimulated controls) in the presence of 1 mg/ml cytochrome C. Superoxide anion release was assessed spectrophotometrically. Values represent mean ± SEM above unstimulated control values (nmol superoxide anion release/10^6 cells: isotonic 3.25 ± 0.54, hypotonic shock 5.30 ± 0.38; hypotonic reversal 2.97 ± 0.67) (n = 3 in triplicate). *P<0.05, significantly different from isotonic conditions (ANOVA).

3.3.1.2 $InsP_6$ Priming of fMLP-Stimulated Neutrophil Shape Change

The effect of $InsP_6$ on basal and fMLP-induced shape change was used as a sensitive indicator of potential chemotactic (Haston and Shields, 1985) and priming (Haslett *et al.,* 1985) activity. A 60 s exposure of neutrophils to $InsP_6$ (100 nM-1mM) had no effect on resting cellular morphology, but caused a concentration-dependent enhancement of 0.1 nM fMLP-induced shape change, that reached a plateau at 100 μM $InsP_6$ (Figure 3.4a): this agrees with the reported priming effect of $InsP_6$ on fMLP-stimulated superoxide anion release (Eggleton *et al.,* 1991). The optimal preincubation time for priming of fMLP-induced shape change was subsequently determined. $InsP_6$ (100 μM) caused a small, but significant, enhancement of fMLP (0.1 nM)-induced shape change in un-primed neutrophils that was maximal after a 30-120 s pre-incubation and spontaneously declined to control levels after 10 min (Figure 3.4b).

A concentration of 0.1 nM fMLP was selected for quantification of the priming effect of $InsP_6$ as this was shown to induce a sub-maximal degree of shape change (approximately 60%) (Figure 3.5), and was therefore potentially amenable to modulation. A 30 s preincubation of neutrophils with 100 μM $InsP_6$ caused a small, but significant, leftwards shift in the concentration-response curve for fMLP-induced shape change, with a decrease in the EC_{50} value from 76 pM to 33 pM.

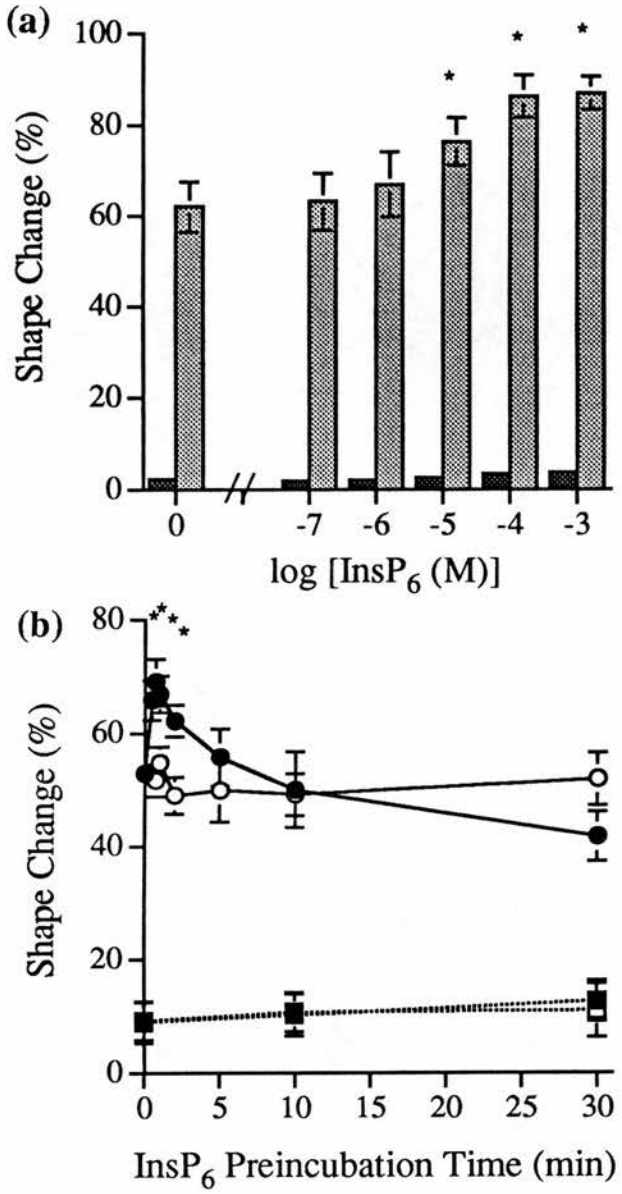


Figure 3.4
Effect of $InsP_6$ on fMLP-Stimulated Neutrophil Shape Change.
(a) Concentration-response for $InsP_6$ priming of fMLP-induced neutrophil shape change. Neutrophils were incubated with $InsP_6$ (0 mM-1 mM, 60 s) prior to stimulation with fMLP (0.1 nM, 10 min, light grey bars) or buffer (dark grey bars). Samples were analyzed by light microscopy. (b) Effect of $InsP_6$ preincubation time on fMLP-induced neutrophil shape change. Neutrophils were pre-incubated for 0.5-30 min with either $InsP_6$ (100 μM, closed symbols) or 25 mM HEPES PBS buffer (pH 7.3, open symbols), prior to treatment with fMLP (0.1 nM, 10 min, circles) or buffer (squares) (mean ± SEM, n = 3 in duplicate). $*P<0.05$, significantly different from fMLP alone (ANOVA).

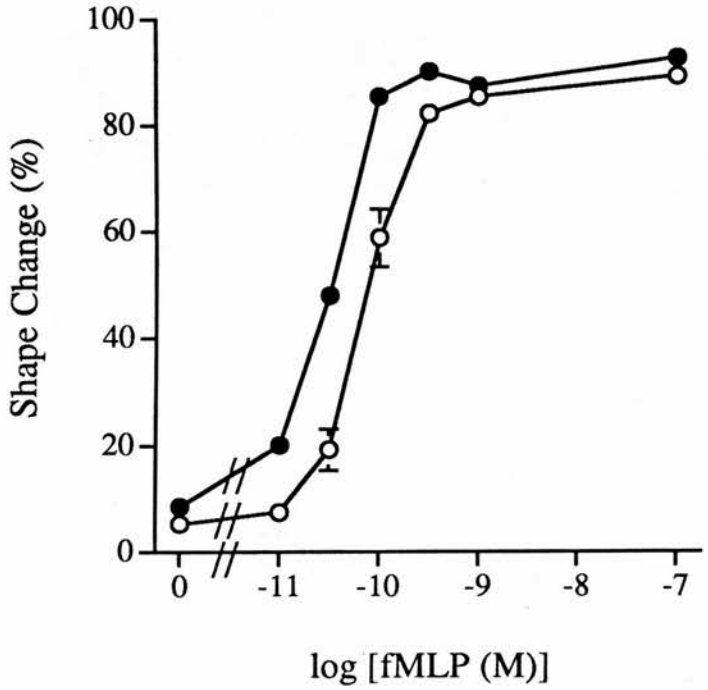


Figure 3.5
Effect of $InsP_6$ on fMLP Concentration-Response Curve for Neutrophil Shape Change.
Neutrophils were incubated with $InsP_6$ (100 μM, 30 s, closed symbols) or 25 mM HEPES PBS buffer (pH 7.3, open symbols), prior to stimulation with fMLP (0-100nM, 10 min). Reactions were terminated at the appropriate times by the addition of 1 ml 2.5% glutaraldehyde and samples were analyzed by light microscopy. (Mean ± SEM of triplicate determination of representative experiment from 5. Where not shown, SEM values fall within symbols).

3.3.1.3 $InsP_6$ Priming of fMLP-Stimulated Superoxide Anion Generation

An optimal $InsP_6$ preincubation time of 30 s was selected for investigation into the priming of fMLP-stimulated superoxide anion release, as the above time-course for priming of the shape change response was similar to that previously reported for priming of respiratory burst activity (Eggleton *et al.*, 1991). The ability of $InsP_6$ to enhance fMLP-stimulated superoxide anion release was compared to that of the well-established neutrophil priming agent LPS (100 ng/ml plus 1% heat-inactivated autologous serum, 60 min) (Condliffe *et al.*, 1996). $InsP_6$ alone (100 μM, 30 s) did not elicit superoxide anion release but significantly enhanced the superoxide anion response induced by fMLP: however, the degree of priming induced by $InsP_6$ was small (1.8 ± 0.3 fold) in comparison to LPS (6.8 ± 0.6 fold) (Figure 3.6), although it agreed with a previous report where $InsP_6$ (250 μM, 5 min) caused a 100-200% priming of the respiratory burst activity induced by fMLP, PMA and opsonised zymosan (Eggleton *et al.*, 1991).

3.3.1.4 Effects of $InsP_6$ on Neutrophil Adhesion Molecule Expression

The shedding of L-selectin and up-regulation of the β_2-integrins have been shown to be a very sensitive indicator of neutrophil priming (Condliffe *et al.*, 1996). Furthermore, $InsP_6$ has been reported to inhibit L-selectin-mediated adherence of neutrophils to activated endothelial cells (Cecconi *et al.*, 1994). Therefore, we investigated the ability of $InsP_6$ to modulate the expression of L-selectin and the β_2-integrins CD11a and CD11b. $InsP_6$ (100 μM, 30 s) did not cause any significant changes in the surface expression of L-selectin, CD11a or CD11b (Figure 3.7), despite fMLP eliciting the down-regulation of L-selectin and the up-regulation of CD11b, as reported previously (Derian *et al.*, 1995; Borregaard *et al.*, 1994).

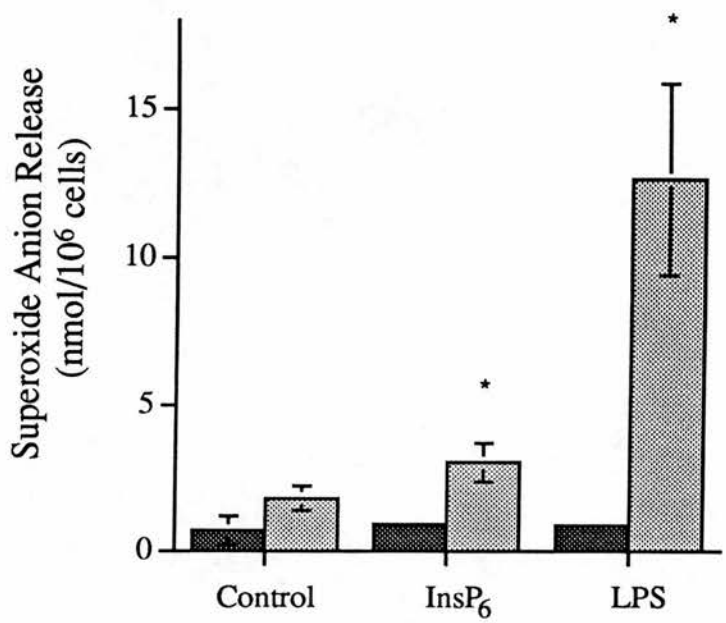


Figure 3.6
Effects of $InsP_6$ and LPS on fMLP-Stimulated Superoxide Anion Release in Human Neutrophils.
Neutrophils were incubated with $InsP_6$ (100 μM, 30 s), LPS (100 ng/ml plus 1% heat-inactivated autologous serum, 60 min) or buffer control, prior to treatment with fMLP (100 nM, 10 min, light grey bars) or buffer control (dark grey bars) in the presence of 1 mg/ml cytochrome C. Superoxide anion release was assessed spectrophotometrically (mean ± SEM, n = 10 in triplicate). $*P<0.05$, significantly different from fMLP alone (ANOVA).

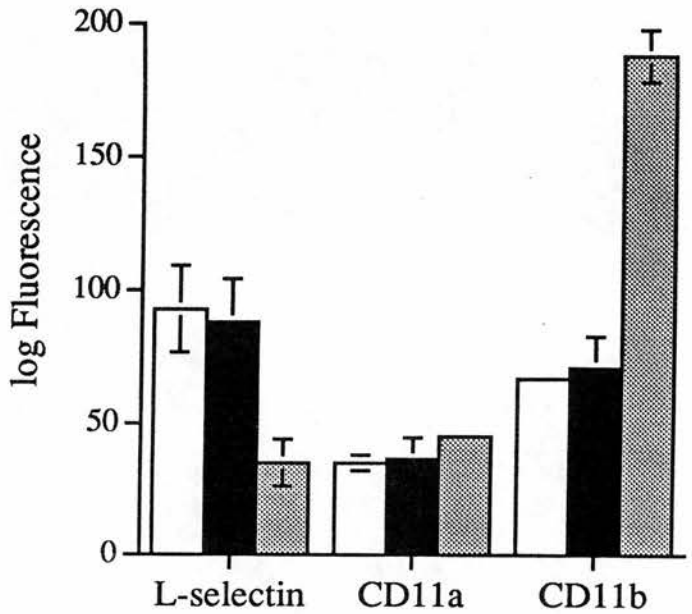


Figure 3.7
Effects of $InsP_6$ on Neutrophil Adhesion Molecule Expression.
Neutrophils were incubated with $InsP_6$ (100 μM, 30 s, black bars), fMLP (100 nM, 10 min, grey bars) or buffer (10 min, white bars). Reactions were stopped at the appropriate times by placing the cells on ice. Neutrophils were then incubated (30 min, 4 °C) with mouse monoclonal antibody to L-selectin, CD11a or CD11b, and subsequently with FITC-conjugated rabbit anti-mouse immunoglobulin. Mean fluorescence was measured by flow cytometry. (Mean ± SEM, n = 4 in duplicate. Where not shown S.E.M. values <2% mean and fall within symbols).

3.3.1.5 Summary of The Functional Effects of $InsP_6$ in Human Neutrophils

(i) $InsP_6$ acts as a neutrophil priming agent *in vitro*, enhancing both the superoxide anion and shape change responses to fMLP. However, these priming effects are small in comparison to the well-established pro-inflammatory mediator, LPS.

(ii) $InsP_6$ alone has no effect on resting neutrophil morphology or adhesion molecule expression, in contrast to more conventional priming agents (see Chapter 4).

(iii) The priming of fMLP-induced shape change is maximal after a 30 s pre-incubation with $InsP_6$, with a subsequent spontaneous reversal that is complete within 10 min. This reversibility is similar to that reported for priming of the fMLP-stimulated superoxide anion response and assembly of cortical F-actin by $InsP_6$, and thus may represent de-priming of a range of functional responses.

3.3.2 [^{3}H]$InsP_6$ Binding to Human Neutrophil Membranes

Owing to the small and unusual nature of the priming effects of $InsP_6$, it was important to ascertain their receptor-dependence. Specific $InsP_6$ binding sites have been identified in a number of mammalian tissues, and the $InsP_6$ receptor from rat cerebellum has now been characterized in some detail (Hawkins *et al.,* 1990; Voglmaier *et al.,* 1992). However, it remains unknown whether human neutrophils possess a similar $InsP_6$ receptor through which to mediate the priming effects of $InsP_6$.

3.3.2.1 Verification of [^{3}H]$InsP_6$ Binding Sites in Rat Cerebellar Membranes

In order to verify the protocol of Hawkins *et al.* (1990), we repeated the examination of [^{3}H]$InsP_6$ binding to rat cerebellar membranes. We observed specific [^{3}H]$InsP_6$ binding as previously reported, that was inhibited in the presence of excess (100 μM) unlabelled $InsP_6$ (Figure 3.8). The IC_{50} for competition with unlabelled $InsP_6$ was 6 nM, but analysis of the curve gave a Hill coefficient significantly less than unity (n_H = 0.62), suggesting the presence of multiple [^{3}H]$InsP_6$ recognition sites. Resolution of the curve into two components, showed that 82% of the [^{3}H]$InsP_6$ bound to a site with a K_i of 4 nM, whilst the remainder bound with lower affinity (K_i = 1 μM). These data agree with previous reports of multiple, although slightly lower-affinity [^{3}H]$InsP_6$ binding sites in rat cerebellum, where 88% of the [^{3}H]$InsP_6$ bound to a site with a K_i of 61 nM, and the remainder had a K_i of 53 μM (Hawkins *et al.,* 1990).

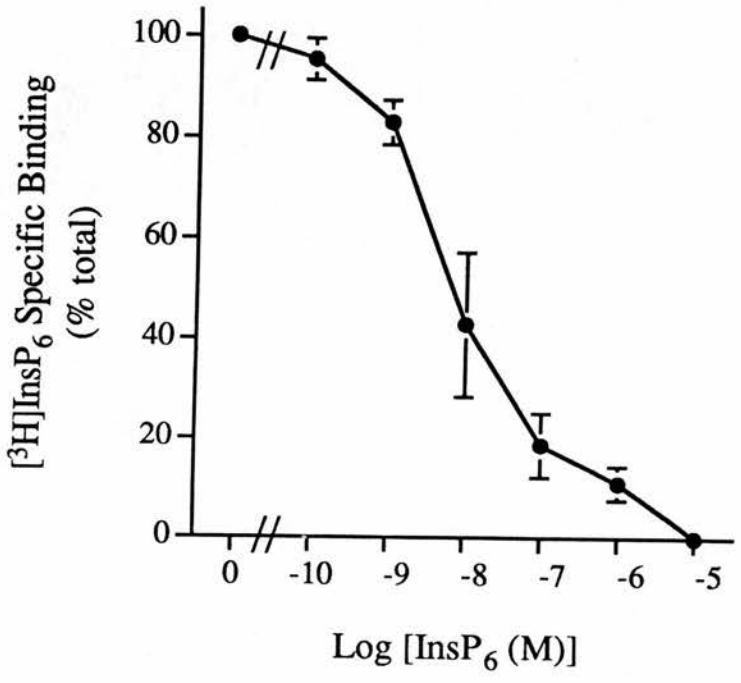


Figure 3.8
Displacement of [^{3}H]$InsP_6$ Binding to Rat Cerebellar Membranes by $InsP_6$.
Assays were performed with 2.5 nM [^{3}H]$InsP_6$, 0.1 mg rat cerebellar membrane protein and increasing concentrations of $InsP_6$ in NKET buffer. Incubations were performed for 90 min at 4 °C, with separation of bound from free radioligand by centrifugation. Non-specific binding was determined in the presence of 100 μM unlabelled $InsP_6$ (mean ± S.E.M., n = 4 in duplicate).

3.3.2.2 Kinetics of [^{3}H]InsP$_6$ Binding to Human Neutrophil Membranes

Having verified the presence of [^{3}H]InsP$_6$ binding sites in rat cerebellar membranes, we proceeded with the examination of [^{3}H]InsP$_6$ binding to human neutrophil membranes. The total binding of [^{3}H]InsP$_6$ (2.5 nM, approximately 90,000 d.p.m.) to human neutrophil membranes containing 0.1 mg protein/ml, was approximately 3,500 d.p.m. (200 fmol/mg protein) and reached equilibrium by 90 min (Figure 3.9). Corresponding incubations in the presence of excess (100 μM) unlabelled InsP$_6$ yielded NSB values of approximately 300 d.p.m. (i.e. <10% of total [^{3}H]InsP$_6$ binding). [^{3}H]InsP$_6$ binding to neutrophil membranes was reversible after 90 min, with addition of 100 μM unlabelled InsP$_6$ causing a rapid displacement (within 2 min) of >80% of the bound radioactivity (Figure 3.9).

3.3.2.3 Saturation of [^{3}H]InsP$_6$ Binding to Neutrophil Membranes

[^{3}H]InsP$_6$ binding failed to saturate up to a radioligand concentration of 10 nM. Owing to both the expense of radiolabelled compounds and the knowledge of the calculated K_i values (150 nM and 5 μM) for [^{3}H]InsP$_6$ binding to neutrophil membranes, attempts to fully saturate [^{3}H]InsP$_6$ binding were not taken further.

3.3.2.4 Competition of [^{3}H]InsP$_6$ Binding to Neutrophil Membranes

Analysis of the competition for [^{3}H]InsP$_6$ binding by unlabelled InsP$_6$ (Figure 3.10a) gave a Hill coefficient significantly less than unity (n_H = 0.55). This value, together with the demonstration of a curvilinear Bound versus *Bound × Inhibitor* plot (Figure 3.10b), suggested the presence of at least two [^{3}H]InsP$_6$ binding sites in human neutrophil membranes. Resolution of the InsP$_6$ competition curve into two components, gave 53% of the [^{3}H]InsP$_6$ binding to a site of K_i 150 nM and 47% to a lower affinity site of K_i 5 μM. This differs from the [^{3}H]InsP$_6$ binding we observed, under identical assay conditions, in rat cerebellar membranes, where the majority of [^{3}H]InsP$_6$ bound to a higher affinity site of K_i 4 nM and the remainder bound with a K_i of 1 μM. For comparison, the previously reported [^{3}H]InsP$_6$ binding in

membranes from rat cerebellum, rat cerebral hemispheres, rat anterior pituitaries, rat heart, and bovine adrenal chromaffin cells has respective K_D values of 61 nM (Hawkins *et al.,* 1990), 33 nM (Nicoletti *et al.,* 1990), 91 nM (Nicoletti *et al.,* 1990), 30 nM (Rowley *et al.,* 1996) and 90 nM (Regunathan *et al.,* 1992); the purified rat cerebellar receptor is of higher affinity, with a K_D of 14 nM (Theibert *et al.,* 1992).

3.3.2.5 Specificity of [^{3}H]$InsP_6$ Binding to Neutrophil Membranes

In order to establish the specificity of [^{3}H]$InsP_6$ binding to human neutrophil membranes, competition experiments were performed using unlabelled Ins(1,3,4,5,6)P_5 and Ins(1,4,5)P_3 (Figure 3.11), which have previously been found to displace $InsP_6$ from its receptors in other tissue preparations (Nicoletti *et al.,* 1990; Hawkins *et al.,* 1990). As the inhibition curve for Ins(1,3,4,5,6)P_5 had a Hill coefficient significantly less than unity, it was best resolved into two components, where 40% of sites had an IC_{50} of 423 nM and the rest an IC_{50} of 18 μM, whilst Ins(1,4,5)P_3 bound with even lower affinity (IC_{50} 30 μM). Thus, [^{3}H]$InsP_6$ binding in human neutrophil membranes displays only a 3-fold selectivity for $InsP_6$ over Ins(1,3,4,5,6)P_5. This specificity for $InsP_6$ is less than that observed in other mammalian tissues, where lower inositol phosphates bind to $InsP_6$ receptors with a reduced affinity of at least one order of magnitude (Nicoletti *et al.,* 1990; Hawkins *et al.,* 1990). However, the purified $InsP_6$ receptor from rat cerebellum displays only a two-fold selectivity for $InsP_6$ over Ins(1,3,4,5,6)P_5 (Theibert *et al.,* 1992).

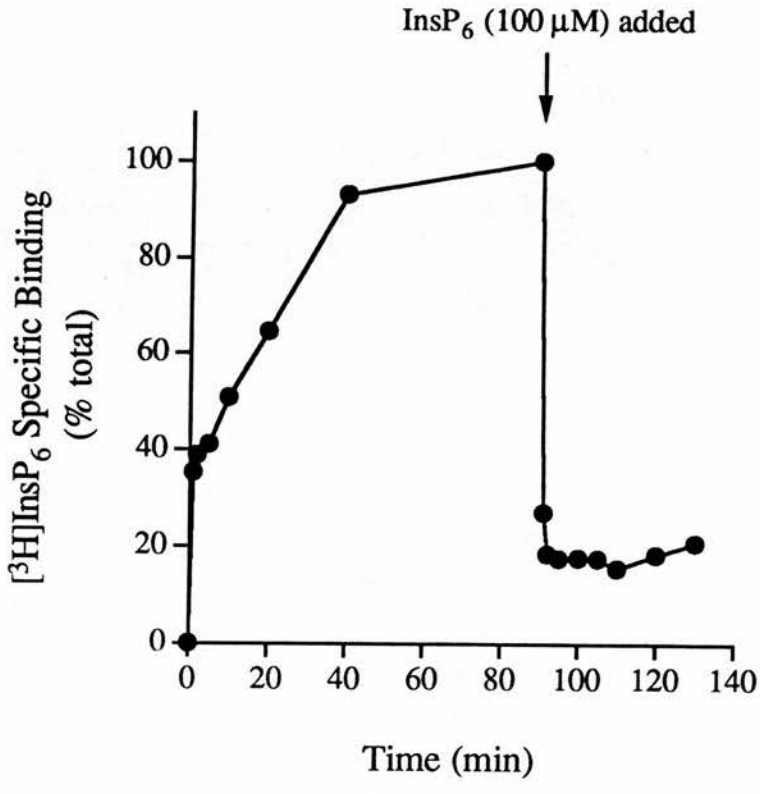


Figure 3.9
Kinetics of [^{3}H]$InsP_6$ Binding to Human Neutrophil Membranes.
Incubations were performed for 0-90 min with 2.5 nM [^{3}H]$InsP_6$ and 0.1 mg human neutrophil membrane protein in NKET buffer (4 °C). 100 μM unlabelled $InsP_6$ was added after 90 min, and incubations continued for a further 40 min. Bound and free radioligand were separated by centrifugation (mean of duplicate determination from representative experiment of 4).

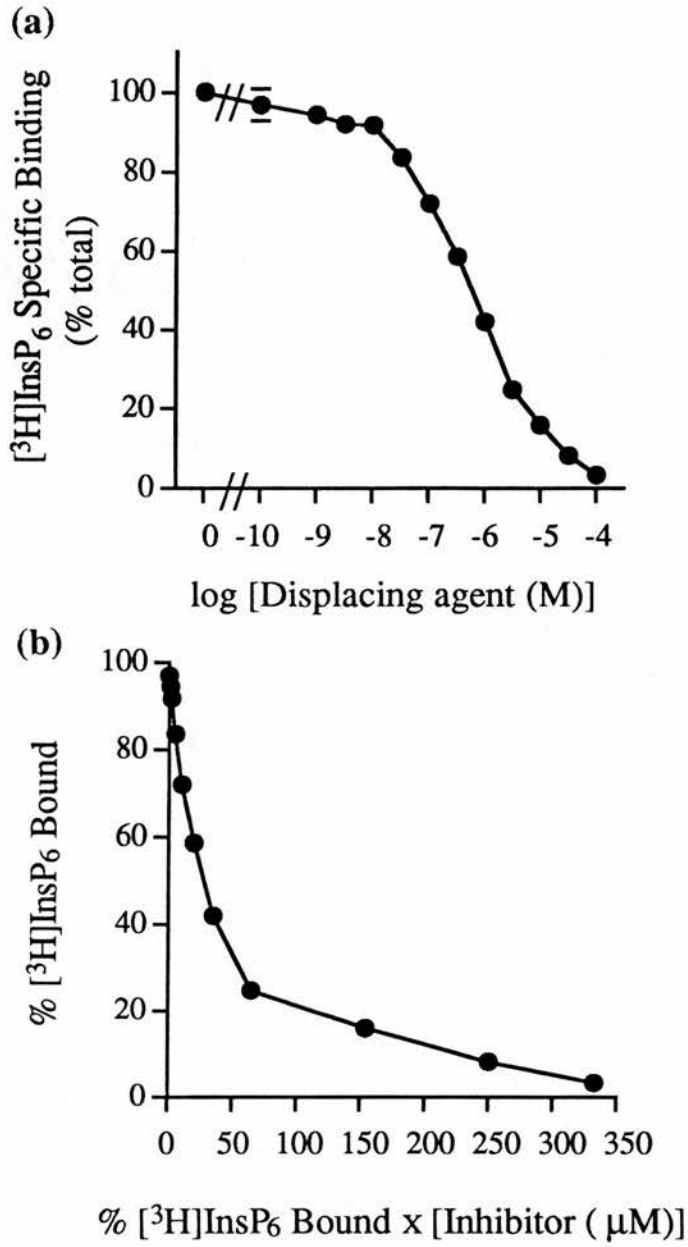


Figure 3.10

(a) Displacement of [^{3}H]$InsP_6$ Binding to Human Neutrophil Membranes by $InsP_6$.

(b) Bound versus *Bound* × *Inhibitor* Plot for Competition of [^{3}H]$InsP_6$ Binding by $InsP_6$.

Assays were performed with 2.5 nM [^{3}H]$InsP_6$, 0.1 mg human neutrophil membrane protein, and increasing concentrations of $InsP_6$ in NKET buffer. Incubations were performed for 90 min at 4 °C, with separation of bound from free radioligand by centrifugation. Non-specific binding was determined in the presence of 100 μM unlabelled $InsP_6$. (Mean ± S.E.M., n = 8 in duplicate. Where not shown, S.E.M. <2% mean and fall within symbols).

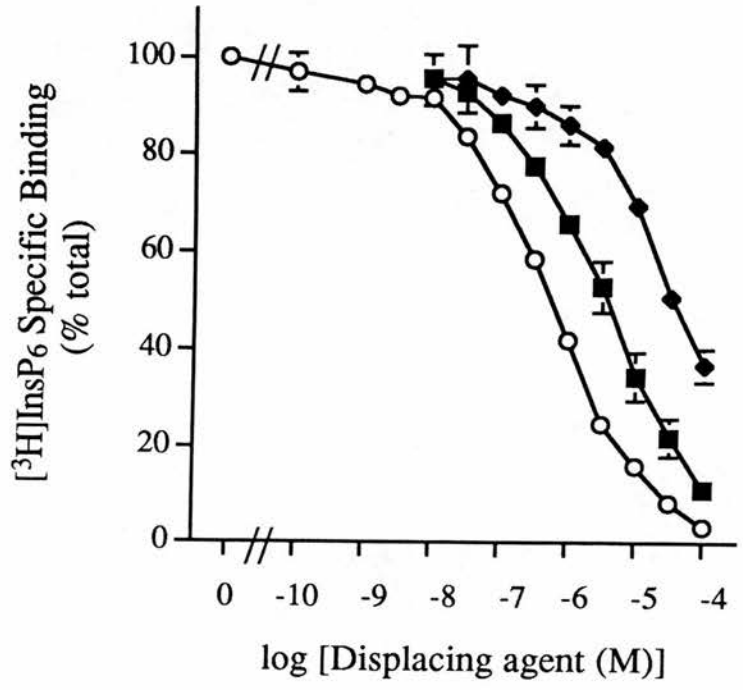


Figure 3.11
Displacement of [^{3}H]$InsP_6$ Binding to Human Neutrophil Membranes by $InsP_6$, $Ins(1,3,4,5,6)P_5$ and $Ins(1,4,5)P_3$.
Assays were performed with 2.5 nM [^{3}H]$InsP_6$, 0.1 mg human neutrophil membrane protein, and increasing concentrations of $InsP_6$ (white circles), $Ins(1,3,4,5,6)P_5$ (black squares), and $Ins(1,4,5)P_3$ (black diamonds) in NKET buffer. Incubations were performed for 90 min at 4 °C, with separation of bound from free radioligand by centrifugation. Non-specific binding was determined in the presence of 100 μM unlabelled $InsP_6$. (Mean ± S.E.M., n = 8 in duplicate. Where not shown, S.E.M. <2% mean and fall within symbols).

3.3.2.6 Metabolism of [^{3}H]$InsP_6$

We have demonstrated that inositol phosphates other than $InsP_6$ are able to associate, albeit with lower affinity, to the [^{3}H]$InsP_6$ binding sites in human neutrophil membranes. Therefore, the possibility arose that [^{3}H]$InsP_6$ metabolites generated during the 90 min incubation period may have confounded the measurement of [^{3}H]$InsP_6$ binding, and contributed to its apparent multi-site nature (see figure 3.10b). H.P.L.C. analysis of post-incubation supernatants demonstrated a start radioligand purity of >99.9%, with no detectable [^{3}H]$InsP_6$ metabolism ([^{3}H]$InsP_{1\text{-}5}$) during the 90 min incubation period (Figure 3.12). This agrees with the metabolic stability of [^{3}H]$InsP_6$ previously reported under identical assay conditions, using rat cerebellar membranes (Hawkins *et al.,* 1990).

3.3.2.7 Protein Dependence of [^{3}H]$InsP_6$ Binding to Neutrophil Membranes

[^{3}H]$InsP_6$ binding was directly proportional to the protein content of the membranes up to 0.2 mg protein/ml, until saturation was achieved at 0.5 mg protein/ml (Figure 3.13). This contrasts with the failure to saturate [^{3}H]$InsP_6$ binding in both rat cerebral hemisphere (Nicoletti *et al.,* 1990) and rat cerebellar membranes (Hawkins *et al.,* 1990), at protein concentrations $\leq$ 2 mg/ml. In a separate series of experiments, the effect of protein denaturation on [^{3}H]$InsP_6$ binding was assessed by boiling the membranes for 90 min prior to use. This caused a >90% reduction in specific [^{3}H]$InsP_6$ binding (data not shown), as previously observed in membranes derived from rat cerebral hemispheres (Nicoletti *et al.,* 1990). This suggests that the majority of [^{3}H]$InsP_6$ binds to a protein component of the membrane: the remainder could represent a non-protein interaction, including an increase in the NSB to membranes disrupted by prolonged boiling.

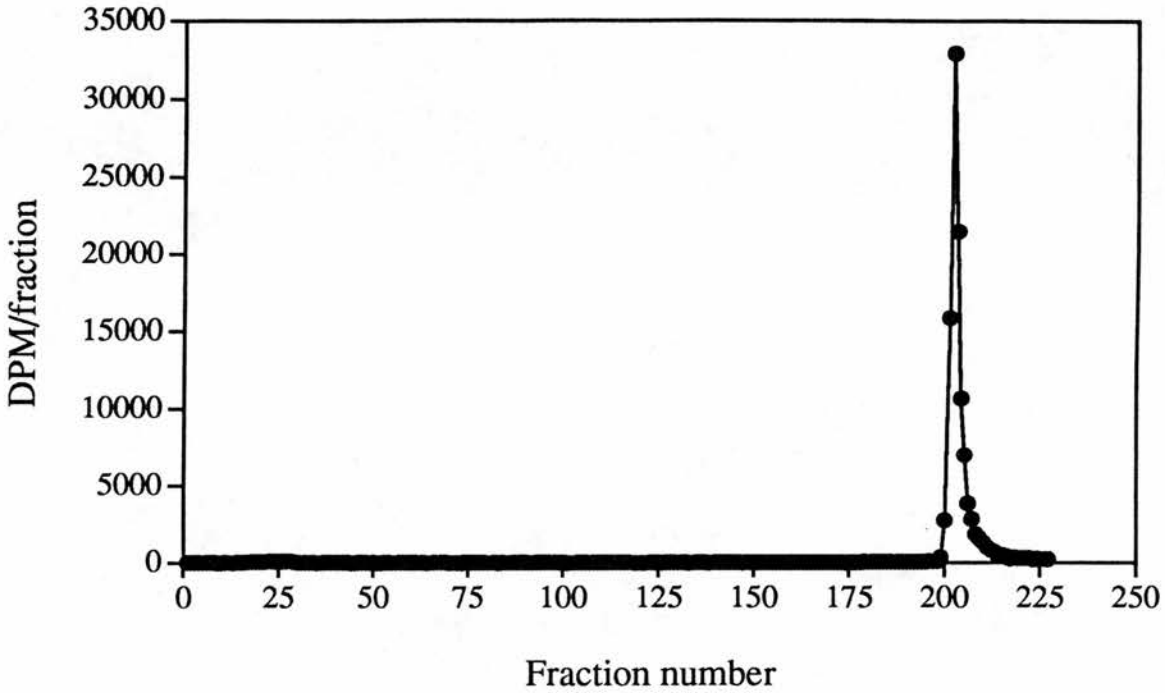


Figure 3.12
HPLC Profile of [^{3}H]InsP$_6$.
[^{3}H]InsP$_6$ (specific activity 15-24 Ci/mmol) was analyzed by HPLC (eluted with an ammonium formate-H_2O gradient, see 3.2.5) and shown to be free of lower inositol phosphate contaminants.

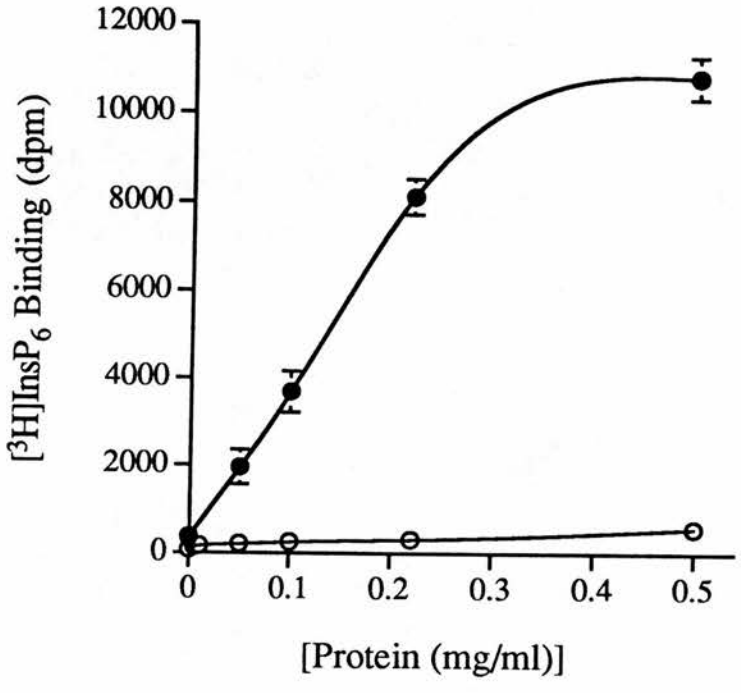


Figure 3.13
Protein Dependence of [^{3}H]$InsP_6$ Binding to Human Neutrophil Membranes.
Assays were performed with 0-0.5 mg human neutrophil membrane protein and 2.5 nM [^{3}H]$InsP_6$ in NKET buffer (black cicles). Incubations were performed for 90 min at 4 °C with separation of bound from free radioligand by centrifugation. Non-specific binding (white circles) was determined in the presence of 100 μM unlabelled $InsP_6$. (Mean ± S.E.M., n = 4 in duplicate. Where not shown, S.E.M. <2% mean and fall within symbols).

3.3.2.8 Modulation of [^{3}H]InsP$_6$ Binding to Neutrophil Membranes by Mono- and Divalent Cations

It has previously been shown that micromolar quantities of various di- and tri-valent cations (e.g. Mg^{2+}, Ca^{2+}, Cu^{2+}, Al^{3+}, Fe^{3+}), but not mono-valent cations (e.g. Na^+), can increase specific [^{3}H]InsP$_6$ binding to membranes prepared from rat cerebellum (Poyner *et al.,* 1993; Cooke *et al.,* 1991), rat cerebral hemispheres and cultured cerebellar neurones (Nicoletti *et al.,* 1990). Therefore, we examined the influence of various mono- and di-valent cations upon [^{3}H]InsP$_6$ binding to human neutrophil membranes. For each buffer condition, total [^{3}H]InsP$_6$ binding was compared to that obtained in NKET buffer, with this value referred to as 100% binding (Figure 3.14). Omission of 5 mM EDTA (a chelator of both Ca^{2+} and Mg^{2+}) increased total binding by 158 ± 20%. Replacement of EDTA with 5 mM EGTA (a Ca^{2+} chelator) caused a 76 ± 25% increase in binding, with a further increase of 89 ± 3% seen upon addition of 1 mM Mg^{2+}. [^{3}H]InsP$_6$ binding was also affected by variations in Na^+ and K^+ concentration: an increase of 79 ± 18%, and a decrease of 61 ± 6%, was observed in KCl-free and NaCl-free buffer, respectively. Thus, the cations Ca^{2+}, Mg^{2+} and Na^+ enhance, whereas K^+ inhibits, [^{3}H]InsP$_6$ binding to human neutrophil membranes.

NSB was similar under all conditions studied (7.3 ± 0.8% of total binding), except in the presence of 5 mM EGTA plus 1 mM $MgCl_2$ when there was a dramatic increase in membrane pellet-associated [^{3}H]InsP$_6$ (52,014 ± 4,362 d.p.m. i.e. approximately 70% of the total [^{3}H]InsP$_6$ added). This would suggest the formation of insoluble [^{3}H]InsP$_6$-Mg^{2+} precipitates, as previously observed with 1 mM Ca^{2+} (Nicoletti *et al.,* 1990) and Fe^{3+} concentrations up to 10 μM (Poyner *et al.,* 1993).

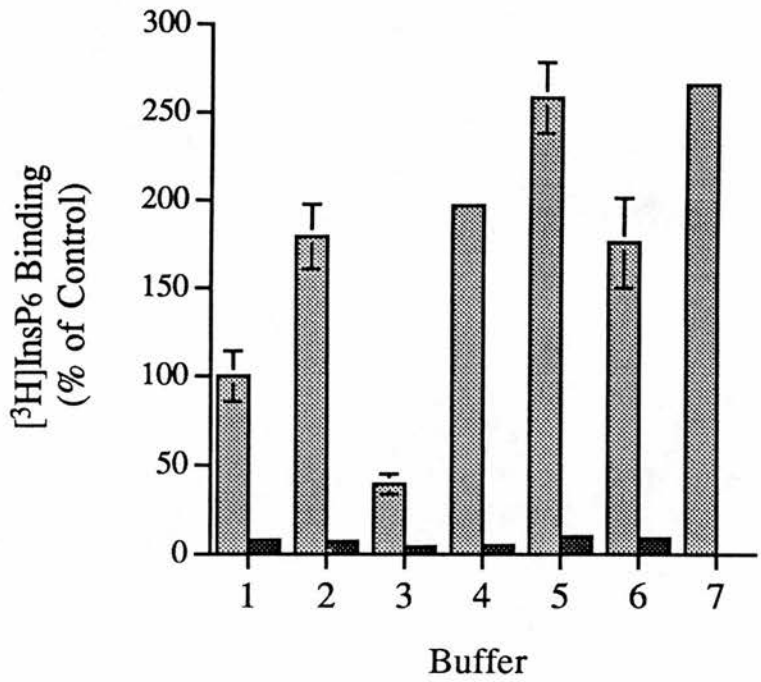


Figure 3.14
Effects of Mg^{2+}, Ca^{2+}, Na^{+}, and K^{+} on [^{3}H]$InsP_6$ Binding to Human Neutrophil Membranes.

Neutrophils were resuspended in a series of 20 mM Tris/HCl buffers (pH 7.7) containing: 5 mM EDTA, 100 mM KCl, 20 mM NaCl (column 1); 5 mM EDTA, 20 mM NaCl (column 2); 5 mM EDTA, 100 mM KCl (column 3); 5 mM EDTA (column 4); 100 mM KCl, 20 mM NaCl (column 5); 5 mM EGTA, 100 mM KCl, 20 mM NaCl (column 6); 5 mM EGTA, 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$ (column 7). Neutrophils were then homogenized, pelleted, and resuspended in the same series of buffers at 0.1 mg membrane protein/ml. [^{3}H]$InsP_6$ binding to the membranes was determined using 2.5 nM [^{3}H]$InsP_6$ for 90 min at 4 °C (in the absence, light grey bars, or presence of 100 μM unlabelled $InsP_6$, NSB, dark grey bars). (Mean ± S.E.M., n = 3 in duplicate. Where not shown, S.E.M. <2% mean and fall within symbols). 100% binding represents 4,958 ± 197 d.p.m., and NSB in buffer 7 was >70% total [^{3}H]$InsP_6$ added.

3.3.2.9 pH-dependence of [^{3}H]$InsP_6$ Binding to Neutrophil Membranes

Characterization of the purified $InsP_6$ receptor from rat cerebellum revealed that [^{3}H]$InsP_6$ binding had a symmetrical pH-dependence curve, with maximal binding at pH 6.0-7.0 (Theibert *et al.,* 1992; Theibert *et al.,* 1991). This pH-dependence was also observed with membranes derived from rat cerebral hemispheres (Nicoletti *et al.,* 1990). Therefore, we investigated whether [^{3}H]$InsP_6$ binding to human neutrophil membranes displayed a similar pH-dependence. In contrast to observations in rat brain, specific [^{3}H]$InsP_6$ binding was markedly enhanced under alkaline conditions, with a peak of maximal binding at pH 8.0 (750 fmol/mg protein) (Figure 3.15). Below pH 7.0 [^{3}H]$InsP_6$ binding remained low. NSB was similar at all pH values studied (313 ± 24 d.p.m.).

To verify that alterations in pH were not causing [^{3}H]$InsP_6$ binding to the Eppendorf tubes, the experiment was repeated in the absence of membranes. The total [^{3}H]$InsP_6$ binding recorded to non-membrane components, at all pH values, was equivalent to the NSB determined in the presence of membranes (data not shown).

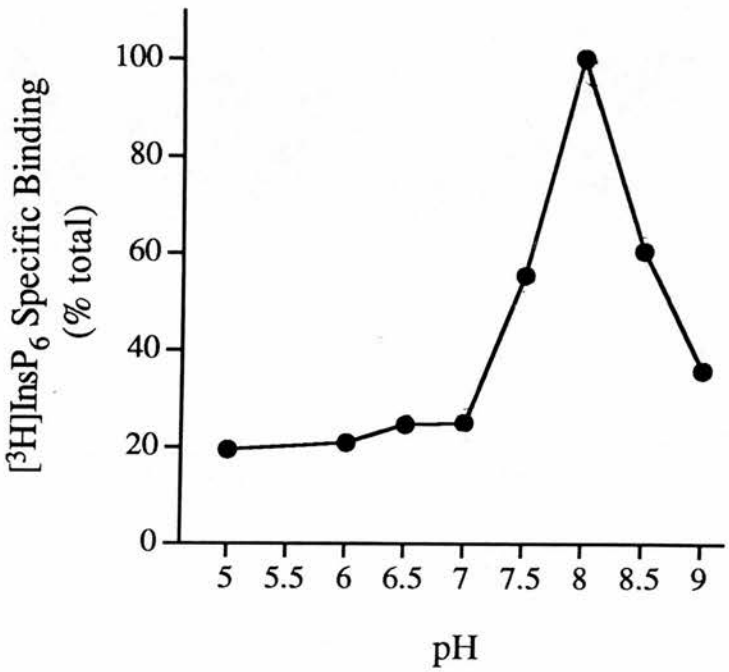


Figure 3.15
pH-Dependence of Specific [^{3}H]InsP$_6$ Binding to Human Neutrophil Membranes.
[^{3}H]InsP$_6$ binding to human neutrophil membranes was determined using 2.5 nM [^{3}H]InsP$_6$ and 0.1 mg human neutrophil membrane protein in a range of 25 mM Tris (pH 7.5-9.0) and Tris-maleate (pH 5.5-7.0) buffers. Incubations were performed at 4 °C for 90 min, and non-specific binding was determined in the presence of 100 μM unlabelled InsP$_6$ (mean of maximal specific [^{3}H]InsP$_6$ binding (13,355 ± 743 d.p.m), n = 2 in triplicate).

3.3.2.10 Heparin Inhibition of [^{3}H]InsP$_6$ Binding to Neutrophil Membranes

Heparin is a highly-charged glycosaminoglycan whose pyranose ring structures (six carbon saccharide residues) show similarity to the chair conformation of inositol. Heparin has the potential to sterically block and inhibit the binding of inositol derivatives, as seen with the $InsP_6$ and $InsP_4$ receptors in rat cerebellum (Theibert *et al.,* 1992). *Vice versa*, inositol derivatives may inhibit heparin binding, as observed when $InsP_6$ blocks heparin binding to fibroblast growth factor (Morrison *et al.,* 1994). This led us to investigate whether heparin could inhibit [^{3}H]$InsP_6$ binding to human neutrophil membranes. As demonstrated in Figure 3.16, heparin inhibited the binding of [^{3}H]$InsP_6$ in a concentration-dependent manner, with >80% inhibition at a heparin concentration of 1 mg/ml. However, an IC_{50} value of 25 μg/ml suggests that heparin is a less potent inhibitor of [^{3}H]$InsP_6$ binding to human neutrophil membranes than to rat cerebellar membranes, where an IC_{50} of 0.6 μg/ml has been reported (Theibert *et al.,* 1992).

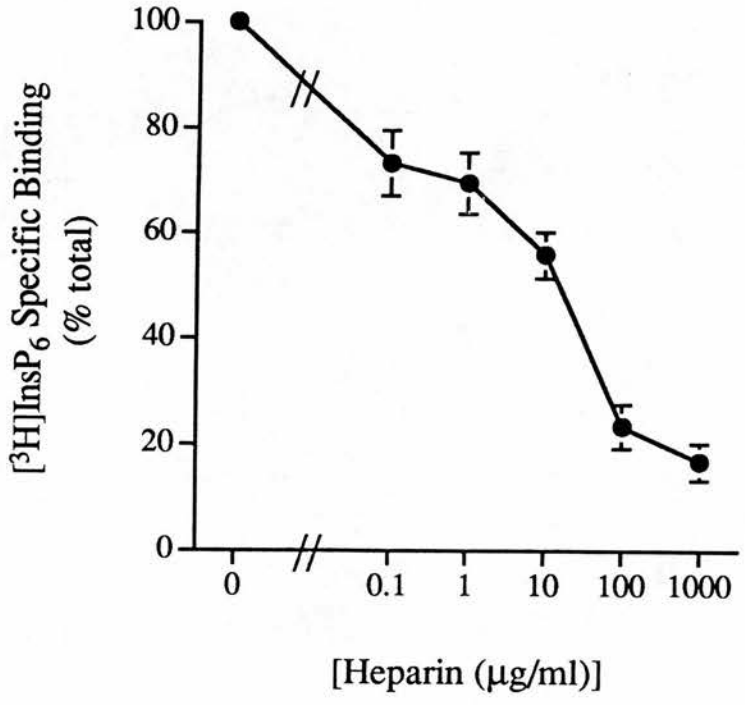


Figure 3.16
Displacement of [^{3}H]$InsP_6$ Binding to Human Neutrophil Membranes by Heparin.
Assays were performed with 2.5 nM [^{3}H]$InsP_6$, 0.1 mg human neutrophil membrane protein and increasing concentrations of heparin (0.1 μg/ml-1 mg/ml) in NKET buffer. Incubations were performed for 90 min at 4 °C, with separation of bound from free radioligand by centrifugation. Non-specific binding was determined in the presence of 100 μM unlabelled $InsP_6$ (mean ± S.E.M., n = 3 in duplicate).

3.3.3 [^{3}H]$InsP_6$ Binding Sites on Intact Human Neutrophils

Although we had demonstrated the presence of $InsP_6$ binding sites in human neutrophil membrane preparations, the above characterization studies suggested they were somewhat atypical when compared to $InsP_6$ receptors identified in other tissues. Thus, if $InsP_6$ was to mediate its priming effects through these binding sites, their location on the extracellular surface of neutrophil plasma membranes was essential. Therefore, the binding of [^{3}H]$InsP_6$ to intact human neutrophils was quantified.

3.3.3.1 Separation of Bound from Free [^{3}H]$InsP_6$ by Simple Centrifugation

The specific binding of [^{3}H]$InsP_6$ to freshly-prepared neutrophils, incubated in either NKET (pH 7.7) or HEPES (25 mM)-buffered PBS (pH 7.5), was markedly reduced (by 78.9 ± 4.2% and 75.7 ± 2.6%, respectively) in comparison to that observed in neutrophil membrane preparations (Figure 3.17a). However, assessment of neutrophil viability, measured by the ability of cells to exclude trypan blue dye, revealed that approximately 10% of the pelleted neutrophils were permeable at the end of the assay.As this method contained three, consecutive centrifugation steps (all at 25 °C, 3,000*g*, 2 min) to separate bound from free radioligand, an alternative separation method was selected using a one-step centrifugation (4 °C, 13,000*g*, 1 min) through an inert silicone oil cushion.

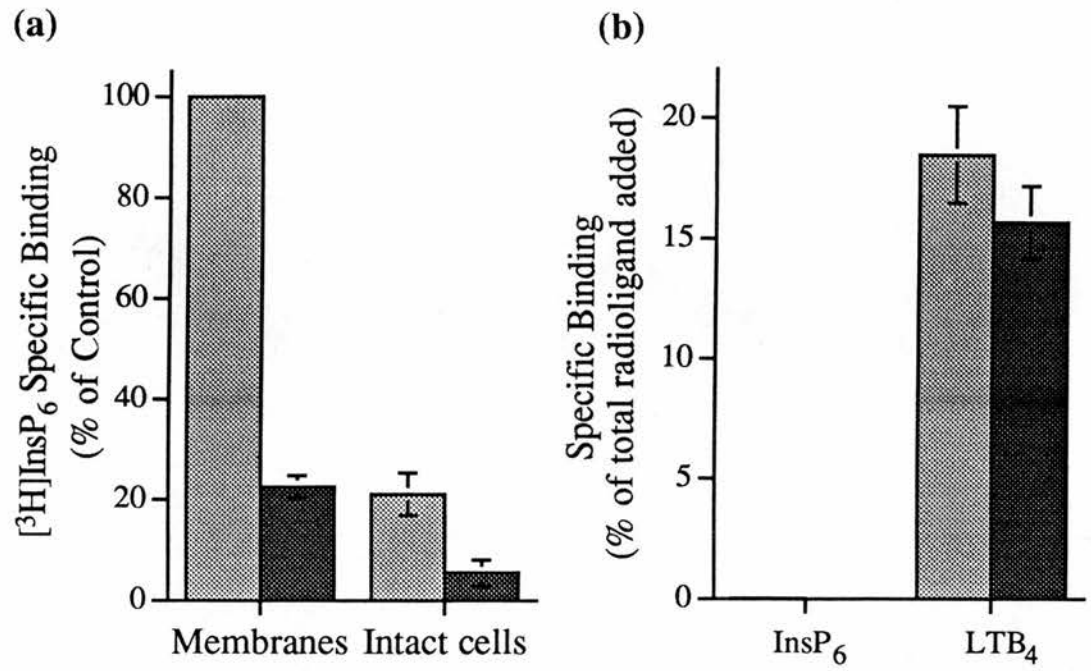


Figure 3.17
[^{3}H]InsP$_6$ Binding to Intact Human Neutrophils.
Assays were performed using intact human neutrophils (3×10^6/ml) or human neutrophil membrane protein (0.1 mg/ml) in NKET buffer (ph 7.7, light bars) or HEPES (25 mM)-buffered PBS (pH 7.5, dark bars). Incubations were performed with 2.5 nM [^{3}H]InsP$_6$ or 10 pM LTB$_4$ (b only) for 90 min at 4 °C. Bound and free radioligand were separated by: (a) centrifugation (3000*g*, 2 min); or (b) centrifugation through a silicone oil cushion (13000*g*,1 min). Non-specific binding was determined in the presence of 100 μM unlabelled InsP$_6$ or 100 nM LTB$_4$ (mean ± S.E.M. for (a) n = 4, or (b) n = 3, each performed in duplicate).

3.3.3.2 Separation of Bound from Free [^{3}H]InsP$_6$ by Centrifugation through an Inert Oil Cushion

This protocol maintained neutrophils at a cell viability of >99.5% throughout the assay period. A negligible amount (<0.03%) of the [^{3}H]InsP$_6$ added was associated with the whole cell pellet, irrespective of the incubation buffer used (Figure 3.17b). This implies that InsP$_6$ does not bind to the extracellular surface of neutrophils. As human neutrophils are known to possess extracellular LTB$_4$ receptors (OFlaherty *et al.,* 1990), this was used as a comparative positive control: >15% of the [^{3}H]LTB$_4$ added to the incubations bound specifically to the surface of neutrophils (Figure 3.17b).

3.3.3.3 Summary of [^{3}H]InsP$_6$ Binding in Human Neutrophils

(i) [^{3}H]InsP$_6$ binding was observed in human neutrophil membranes, that equilibrated after 90 min and dissociated rapidly (2 min). Analysis of the InsP$_6$ displacement data suggested the presence of at least two [^{3}H]InsP$_6$ binding sites: 53% of the sites had a K_I of 150 nM and the remainder were of lower affinity (K_i 5 μM).

(ii) In competition experiments, [^{3}H]InsP$_6$ binding displayed a modest selectivity for InsP$_6$ over Ins(1,3,4,5,6)P$_5$ and InsP(1,4,5)P$_3$.

(iii) [^{3}H]InsP$_6$ binding was optimal at pH 8.0, was enhanced by Ca^{2+}, Mg^{2+} and Na^+, and was inhibited by K^+, heparin, and by pre-boiling the membranes.

(iv) However, [^{3}H]InsP$_6$ binding could not be identified in intact neutrophils.

3.4 Discussion

Having confirmed that the priming of human neutrophils following hypotonic challenge is reversible upon restoration of buffer tonicity, we sought a physiologically-credible mediator that might exhibit reversible, receptor-mediated priming. $InsP_6$ is an abundant, cytosolic inositol polyphosphate (Stuart *et al.,* 1994; Bunce *et al.,* 1993) that has been proposed as a novel neutrophil priming agent, upon its release from necrotic cells within an inflammatory focus (Crawford and Eggleton, 1992; Eggleton *et al.,* 1991). Owing to its ubiquity and potentially transient pro-inflammatory effects, we selected $InsP_6$ for our investigations of reversible priming.

Initial experiments sought to define the capacity of $InsP_6$ to modulate neutrophil functions. Pre-incubation of neutrophils with $InsP_6$ (100 μM) did not affect resting cell morphology, adhesion molecule expression, nor basal NADPH oxidase activity, but caused a small, yet significant, enhancement of both the superoxide anion response (1.8-fold) and polarization response (1.3-fold) to fMLP (100 nM and 0.1 nM, respectively). However, this potentiation of fMLP-induced superoxide anion generation by $InsP_6$ was minor when compared to LPS (6.8 fold), and other established neutrophil priming agents (see Chapter 4). It has previously been reported that $InsP_6$ can prime the respiratory burst activity induced by fMLP, PMA and opsonised zymosan by 100-200% (Eggleton *et al.,* 1991): although we agree quantitatively with respect to fMLP, our 1.8-fold potentiation of the superoxide response by $InsP_6$ sounds far less impressive.

Assessment of the optimal time required for $InsP_6$ to affect neutrophil function, revealed a rapid (maximal at 30-120 s) and transient (terminated by 10 min) enhancement of shape change to fMLP. This correlates well with $InsP_6$-primed superoxide anion generation (Eggleton *et al.,* 1991) and cortical F-actin assembly (Crawford and Eggleton, 1992), which have been reported to have optimal $InsP_6$ pre-incubation times of 30 s and 5 min, with a subsequent 30% reduction by 5 min and

10 min, respectively. However, the lack of a direct effect of $InsP_6$ on resting cell morphology is not in keeping with other etablished priming agents (e.g. PAF and TNFα, see Chapter 4). Therefore, although $InsP_6$ can elicit a rapid and transient priming of certain neutrophil functional responses, these effects are modest and somewhat atypical when compared to more conventional priming agents.

Further studies examined whether the small and transient priming effect of $InsP_6$ was receptor-mediated in the neutrophil. Analysis of [^{3}H]$InsP_6$ binding identified the presence of at least two binding sites in human neutrophil membrane preparations, where $InsP_6$ bound in approximately equal proportions to a site of K_i 150 nM and a lower-affinity site of K_i 5 μM. This was unlike the [^{3}H]$InsP_6$ binding we observed in rat cerebellar membranes, where the majority of [^{3}H]$InsP_6$ bound to a higher affinity site of K_i 4 nM. However, the affinity of this [^{3}H]$InsP_6$ binding was at least one log order of magnitude higher than that previously reported in rat cerebellum (K_i 61 nM), where an identical protocol was followed (Hawkins *et al.,* 1990). The reason for this discrepancy is unknown, but since the purified $InsP_6$ receptor from rat cerebellum has a reported K_D of 14 nM (Theibert *et al.,* 1992), both values obtained from crude membrane preparations lie within the expected range. The $InsP_6$ binding reported in all other membrane preparations has been to a single population of high affinity sites, for example in: rat cerebral hemispheres (K_D 33 nM) (Nicoletti *et al.,* 1990); rat heart (K_D 30 nM) (Rowley *et al.,* 1996); rat anterior pituitaries (K_D 91 nM) (Nicoletti *et al.,* 1990); and bovine adrenal chromaffin cells (K_D 90 nM) (Regunathan *et al.,* 1992). $InsP_6$ binding to these sites was reported to equilibrate faster and dissociate more slowly than in human neutrophil membranes, possibly due to their greater affinity for $InsP_6$. Therefore, as $InsP_6$ binds to several sites in human neutrophil membranes, with lower affinity than that reported in other tissues, the nature of this binding appears different and more complex than in other mammalian cells.

[^{3}H]$InsP_6$ binding to human neutrophil membranes displays a 3-fold selectivity for $InsP_6$ over Ins(1,3,4,5,6)P_5. Although this relatively low specificity is virtually identical to that reported for the purified rat cerebellar $InsP_6$ receptor (Theibert *et al.,*

1992), it contrasts with the greater specificity of $InsP_6$ binding sites reported in rat brain, and bovine adrenal chromaffin cell membrane preparations, where lower inositol phosphates bind with an affinity at least one log order of magnitude less than $InsP_6$ (Regunathan *et al.,* 1992; Nicoletti *et al.,* 1990; Hawkins *et al.,* 1990). However, all the reported binding sites have one thing in common: the more phosphorylated the inositol compound, the higher its affinity for the $InsP_6$ binding site, with a rank order of potency: $InsP_6$ > $Ins(1,3,4,5,6)P_5$ > $Ins(1,3,4,5)P_4$ > $Ins(1,4,5)P_3$ (Hawkins *et al.,* 1990; Regunathan *et al.,* 1992; Theibert *et al.,* 1992).

A pH optimum of 8.0 was observed for [^{3}H]$InsP_6$ binding to human neutrophil membranes, with a marked inhibition at more alkaline values, making a simple charge-based membrane interaction unlikely. This pH-dependency differs from that obtained in rat cerebral cortex (Nicoletti *et al.,* 1990), and rat cerebellum (Theibert *et al.,* 1992), where maximal [^{3}H]$InsP_6$ binding occurred at pH 6 and 7, respectively. Thus, in view of the pH-dependency, specificity, affinity, and heterogeneity of $InsP_6$ binding to human neutrophil membranes, it is unlikely that neutrophil [^{3}H]$InsP_6$ binding sites represent any of those currently identified, including the IGF-II receptor (Kar *et al.,* 1994), the G-protein regulator, arrestin (Palczewski *et al.,* 1991), the Golgi coatomer K^+ channel (Fleischer *et al.,* 1994), or the clathrin assembly proteins, AP-2 (Voglmaier *et al.,* 1992) and AP-3 (Norris *et al.,* 1995). These latter molecules have been identified as potential $InsP_6$ receptors in the rat brain, where they bind to the plasma membrane with clathrin, to form clathrin-coated vesicles (Keen *et al.,* 1979): thus, they may have a role in the internalization of ligand-bound membrane receptors by endocytosis. The AP-2 molecule has been characterized as a complex of four proteins (Theibert *et al.,* 1992), with two doublets of 115 kDa and 105 kDa that bind $InsP_6$ (K_D 14 nM) and two non-binding singlets of 50 and 17 kDa. The high affinity of $InsP_6$ binding to AP-2 implies a physiological role, and $InsP_6$ has been shown to inhibit clathrin-coated vesicle formation (Beck and Keen, 1991).

The ability of cations (Mg^{2+}, Ca^{2+} and Na^+) to potentiate [^{3}H]$InsP_6$ binding in human neutrophil membranes is qualitatively similar to observations (with Mg^{2+}, Fe^{3+} and

Al^{3+}) in rat tissues (Nicoletti *et al.,* 1990; Cooke *et al.,* 1991; Poyner *et al.,* 1993), although no effect of Na^+ was reported. Since cation-potentiated $InsP_6$ binding had been found to be non-saturable and ubiquitous in membranes from rat tissues, it had been suggested that highly-abundant membrane components, such as negatively-charged phospholipid phosphate groups, are more likely to be the site of this binding than a specific membrane protein (Poyner *et al.,* 1993). We also observed this high capacity for $InsP_6$ binding in human neutrophil membranes if 1 mM Mg^{2+} was added with an excess of unlabelled $InsP_6$, as found previously with 1 mM Ca^{2+} (Nicoletti *et al.,* 1990). Thus, multi-valent cations may anchor $InsP_6$ to intracellular membranes, where it may function as: (i) a phosphate store (Berridge and Irvine, 1989); (ii) a cation chelator, by chelating Ca^{2+} and hence regulating intracellular [Ca^{2+}] (Luttrell, 1993), or by chelating Fe^{3+} and acting as an antioxidant (Hawkins *et al.,* 1993); or (iii) an inhibitor of enzymes, including alkaline phosphatase (Martin, 1995), and the $Ins(1,4,5)P_3$/$Ins(1,3,4,5)P_4$ 5- and $Ins(1,3,4,5)P_4$ 3-phosphatases (Hughes and Shears, 1990; Hoer and Oberdisse, 1991).

In contrast, the powerful cation-chelating properties of $InsP_6$ raise the possibility that, in the presence of excess $InsP_6$, insoluble $InsP_6$-cation complexes may form and subsequently precipitate in membranes, as seen with Fe^{3+} concentrations up to 10 μM (Poyner *et al.,* 1993). This precipitation could also explain the "ubiquitous and non-saturable" binding previously reported in rat tissues (Poyner *et al.,* 1993). *In vivo* however, the high intracellular [K^+] might competitively inhibit $InsP_6$-cation complex formation and thus protect against precipitation, as K^+ was found to inhibit $InsP_6$ membrane binding. Also, since >90% of the [^{3}H]$InsP_6$ binding was abolished by pre-boiling the membranes, as shown previously (Nicoletti *et al.,* 1990), binding should represent an association between [^{3}H-$InsP_6$ and specific membrane proteins (if $InsP_6$ is not present in excess). The multi-site nature of $InsP_6$ binding to neutrophil membranes could represent several, discrete binding sites with different affinities for $InsP_6$, or a single population of binding sites capable of exhibiting multiple, interconvertible binding states. It is impossible to predict which of these is correct (with the available data) because the binding characteristics of $InsP_6$ are so complex.

Nevertheless, $InsP_6$ binding to at least one of these sites is affected by alterations in pH and/or cation concentration. These specific $InsP_6$ binding sites in human neutrophil membranes could have a functional role, possibly in the control of endocytosis, akin to $InsP_6$ receptors in rat cerebellum.

In contrast to human neutrophil membrane preparations, [^{3}H]$InsP_6$ binding was not detected in intact neutrophils. Therefore, the transient priming effects of $InsP_6$ are unlikely to be mediated by an $InsP_6$-specific, cell-surface receptor. A variety of non-receptor mechanisms may underly the unusual priming ability of $InsP_6$. The fact that hypotonic shock (Edashige *et al.,* 1993), cell swelling and various negatively-charged agents (Miyahara *et al.,* 1993), can prime the superoxide anion response in neutrophils, whilst lipophilic and positively-charged agents (Miyahara *et al.,* 1993) inhibit superoxide anion generation, implies that an increase in either cell size or the net negative charge across the plasma membrane can prime neutrophils. Thus, the six, negatively-charged phosphate groups of $InsP_6$ may underlie its neutrophil priming effect. However, other inositol polyphosphates do not prime neutrophils (Eggleton *et al.,* 1991), rendering a simple charge-based effect unlikely. $InsP_6$ has been shown to be capable of chelating physiological levels of Ca^{2+}, between the basal intracellular and free extracellular [Ca^{2+}] (Luttrell, 1993). Thus, it would be more plausible to postulate that $InsP_6$ has a non-specific extracellular effect (possibly by acting as a cation chelator), that leads to secondary membrane perturbations, and thus promotes the assembly and subsequent secretagogue stimulation of the membrane-bound NADPH oxidase. These secondary membrane effects could also underlie the priming of superoxide anion generation seen with hypotonic shock and cell swelling.

In conclusion, although human neutrophils possess specific, low affinity [^{3}H]$InsP_6$ binding sites, they are located only on intracellular membranes. This implies that $InsP_6$ may have a functional, intracellular role in neutrophils, possibly as a membrane-bound cation chelator, phosphate store or modulator of endocytosis. In contrast, an extracellular role for $InsP_6$ in modulating neutrophil function is unlikely. Thus, although $InsP_6$ can elicit a rapid and transient priming of human neutrophils,

this relatively small effect does not appear to be mediated by a specific, cell-surface receptor. These findings must, therefore, question the biological significance of $InsP_6$ as a priming agent *in vivo*.

4. CHAPTER 4: DEMONSTRATION OF REVERSIBLE PRIMING OF HUMAN NEUTROPHILS BY PLATELET-ACTIVATING FACTOR

4.1 Introduction

Since the small, yet transient, neutrophil priming effects of $InsP_6$ were not mediated by cell-surface receptors (Chapter 3), and hence may have constituted a non-specific effect, we sought a receptor-mediated priming agent with which to investigate the potential reversibility of neutrophil priming. Allowing for the relative paucity of available literature regarding the kinetics of the priming response, several of the most prominent candidates were discounted in view of their prolonged priming effects in neutrophils, including LPS (Carey *et al.*, 1994; Guthrie *et al.*, 1984; Ichinose *et al.*, 1990), G-CSF (Ichinose *et al.*, 1990), GM-CSF and IFN-γ (Roberts *et al.*, 1993). The remaining pro-inflammatory mediators were graded, depending upon their fulfilment of the following criteria.

The selected agent should primarily:

(i) be an established pro-inflammatory mediator *in vivo*;

(ii) produce a range of effector responses associated with neutrophil priming, including the gold standard priming of agonist-induced superoxide anion generation;

(iii) mediate these effects at physiological concentrations through receptors on the neutrophil surface;

(iv) have previously been reported to induce responses that may be transient.

The three agents that best fitted the above criteria were IL-8, IL-1, and PAF. IL-1 has been reported to enhance the respiratory burst of human neutrophils (Elbim *et al.*, 1994; Sullivan *et al.*, 1989) but, in contrast to G-CSF and LPS, does not retain a

primed superoxide response after 24 hours in culture (Ichinose *et al.*, 1990). In addition, IL-8 has been shown to transiently enhance fMLP-induced responses of neutrophils, including intracellular respiratory burst activity, superoxide anion release, cytochalasin B-dependent arachidonic acid release, and PAF release (Daniels *et al.*, 1992; Roberts *et al.*, 1993). These findings suggest that neutrophil priming induced by certain, receptor-dependent cytokines may be a reversible process. However, the former transient effects of IL-8 were not accompanied by a concomitant reversal of CD11b expression (Roberts *et al.*, 1993). Furthermore, the priming effects of both IL-8 and IL-1 are relatively weak in comparison to other cytokines, such as TNFα and GM-CSF (Elbim *et al.*, 1994; Roberts *et al.*, 1993). Indeed, it was proposed that IL-8 might act predominantly as a regulator of neutrophil adhesion and migration, rather than as a neutrophil priming agent *in vivo* (Roberts *et al.*, 1993); the same might be true for IL-1. Since PAF has well-documented and unquestionable priming effects in human neutrophils, it was selected for our investigations into the potential reversibility of neutrophil priming.

Most *in vitro* studies have investigated the actions of soluble PAF on isolated neutrophils in suspension. However, it should be noted that PAF may also function as a cell-associated, pro-inflammatory mediator, for example when it is co-expressed with P-selectin on the surface of activated endothelial cells (Lorant *et al.*, 1993; Lorant *et al.*, 1991). Although PAF has been shown to prime the respiratory burst elicited by various neutrophil stimulants, including fMLP, PMA and C5a (Pinckard *et al.*, 1992; Dewald and Baggiolini, 1985; Gay *et al.*, 1986), it cannot directly activate this response. The priming of fMLP-induced superoxide anion release is rapid, being maximal within minutes of the PAF addition (Pinckard and Prihoda, 1996; Gay *et al.*, 1986). However, this primed response may not be sustained, since it has been noted that by the end of a 60 min preincubation with 1 μM PAF, the resultant priming of fMLP-stimulated superoxide anion generation had a "tendency to diminish" (Gay *et al.*, 1986). This finding was corroborated by a later report which demonstrated a disappearance of the 10 nM PAF-induced priming effect by 60

min, accompanied by a time-dependent priming of elastase release (Vercellotti *et al.*, 1988).

In view of the predicted, central role of PAF *in vivo* as an initiator of the early pro-inflammatory responses of circulating neutrophils, and the possibility that these actions may contribute to the patho-physiological effects of PAF, this Chapter will assess the potential for reversible PAF-induced neutrophil shape change, CD11b/CD18 acivation, and priming of superoxide anion generation. These observations will be used to determine whether or not neutrophil priming is truly a reversible process.

4.2 Results

4.2.1 Determination of the Optimal Priming Conditions for PAF

Despite abundant demonstrations that PAF can prime human neutrophils, a lack of inter-experimental consistency has produced many conflicting results. These differences may be largely explained by the process of "synergistic priming" which is known to occur between certain priming agents (Roberts *et al.*, 1993; Elbim *et al.*, 1994; Yuo *et al.*, 1991). Thus, if neutrophils were primed at any stage during their isolation from peripheral blood or the subsequent incubation procedure (especially by endogenous LPS), these basally-primed cells may well appear exquisitely sensitive to very low concentrations of priming agents. Therefore, prior to examining the potential for neutrophils to de-prime following PAF treatment, it was necessary to establish the optimal conditions for PAF to elicit its priming effects in human neutrophils.

4.2.1.1 PAF-Induced Priming of fMLP-Stimulated Superoxide Anion Release

Since it has previously been shown that PAF will elicit its maximal priming responses within 0-10 min (Ingraham *et al.*, 1987; Gay *et al.*, 1986; Baggiolini and Dewald, 1986), we selected a 10 min pre-incubation time for initial investigations with PAF. The incubation of human neutrophils with 1 nM-10 μM PAF did not affect spontaneous superoxide anion release, but caused a concentration-dependent enhancement (EC_{50} 50.2 ± 8.4 nM) of the subsequent superoxide response to 100 nM fMLP that reached a plateau with 1 μM PAF (Figure 4.1). This observation is in agreement with several other reports (Pinckard and Prihoda, 1996; Koenderman *et al.*, 1989; Baggiolini and Dewald, 1986; Walker *et al.*, 1991; Gay, 1993).

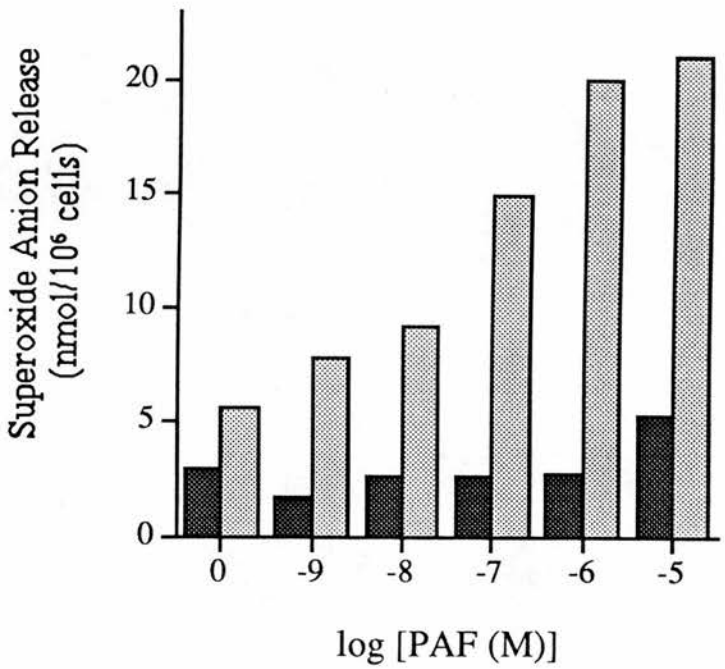


Figure 4.1
Concentration-Response for PAF-Induced Priming of fMLP-Stimulated Superoxide Anion Release in Human Neutrophils.
Neutrophils were incubated with PAF (10 nM-10 μM, 10 min) or buffer control, then treated with fMLP (100 nM, 10 min, light grey bars) or buffer (dark grey bars) in the presence of 1 mg/ml cytochrome C. Superoxide anion release was assessed by scanning spectrophotometry (mean of triplicate determination from representative experiment of 6).

The *priming potential* of PAF was compared quantitatively with that induced by other pro-inflammatory agents used under their optimal priming conditions (Figure 4.2). Thus, whilst PAF (1 μM, 10 min) elicited a 5.5-fold priming of the 100 nM fMLP-stimulated superoxide response, TNFα (200 U/ml, 30 min), LPS (100 ng/ml plus 1% heat-inactivated autologous serum, 60 min) (Alison Condliffe, personal communication), ATP (2 μM, 2 min) (Kuhns *et al.,* 1988), and fMLP (10 nM, 10 min) (Kusner *et al.,* 1991; Bender and Van Epps, 1983; English *et al.,* 1981; Bellavite *et al.,* 1993) elicited priming effects of 6.7-, 6.5-, 1.3-, and 1.6-fold, respectively, with minimal direct activation of superoxide anion release.

4.2.1.2 PAF-Induced Priming of Intracellular Respiratory Burst Activity

As a further index of the relative priming potentials of PAF and TNFα, the DHR oxidation assay was performed as a complementary measure of intracellular respiratory burst activity (Rothe *et al.,* 1991; Royall and Ischiropoulos, 1993; Emmendorffer *et al.,* 1990). Although the respiratory burst activity elicited by either agent alone was small, both PAF (1 μM, 10 min) and TNFα (200 U/ml, 30 min) were able to prime the fMLP response, by 3.8-fold and 10.8-fold, respectively (Figure 4.3).

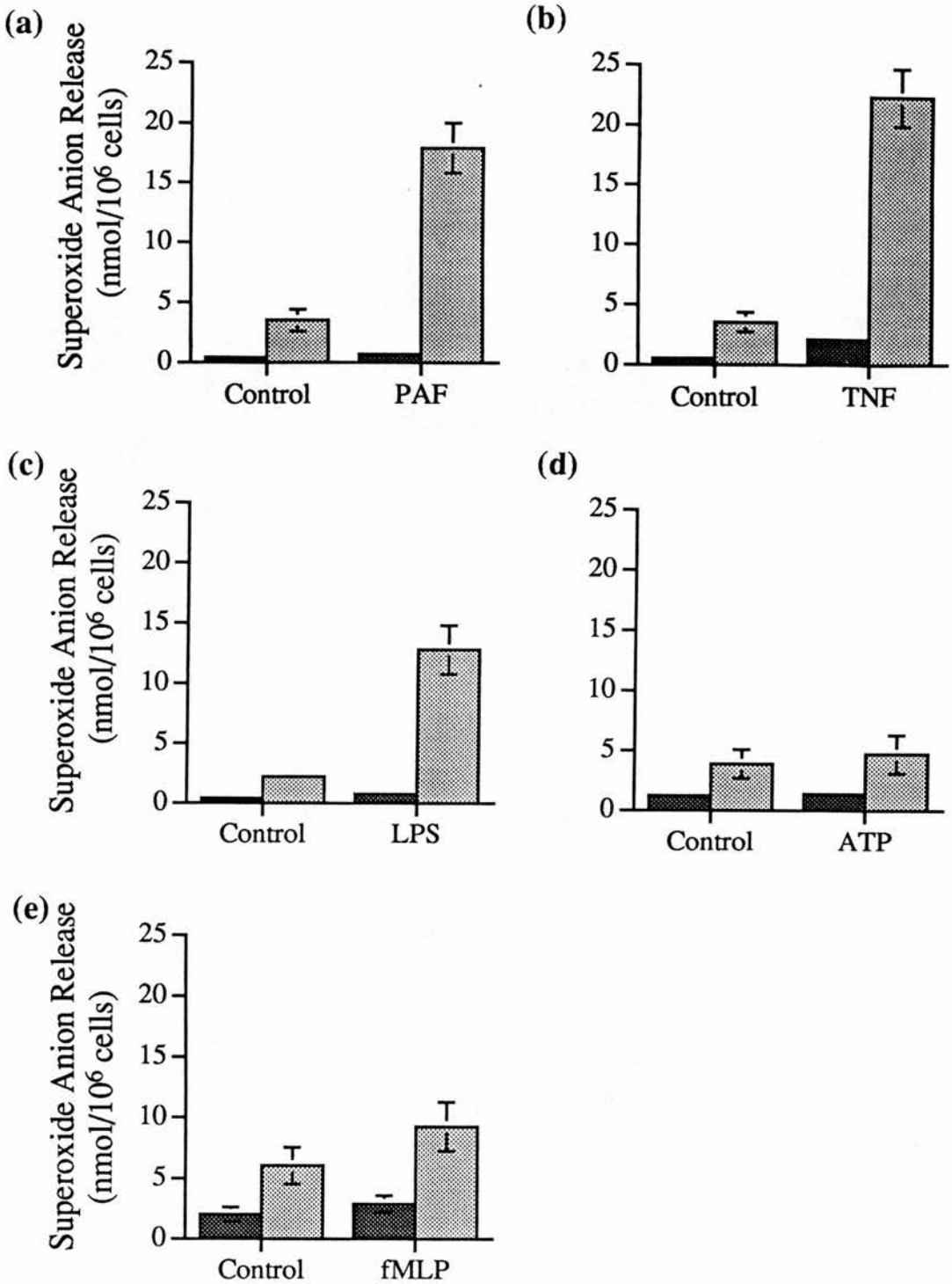


Figure 4.2
The Priming of fMLP-Stimulated Superoxide Anion Release in Human Neutrophils.
Neutrophils were incubated with buffer control or priming agent: (a) PAF (1 μM, 10 min); (b) TNFα (200 U/ml, 30 min); (c) LPS (100 ng/ml plus 1% heat-inactivated autologous serum, 60 min); (d) ATP (2 μM, 2 min); or (e) fMLP (10 nM, 10 min). Neutrophils were then treated with fMLP (100 nM, 10 min, light bars) or buffer control (dark bars) in the presence of 1 mg/ml cytochrome C. Superoxide anion release was assessed by scanning spectrophotometry (mean ± S.E.M., n = 3-6 in triplicate).

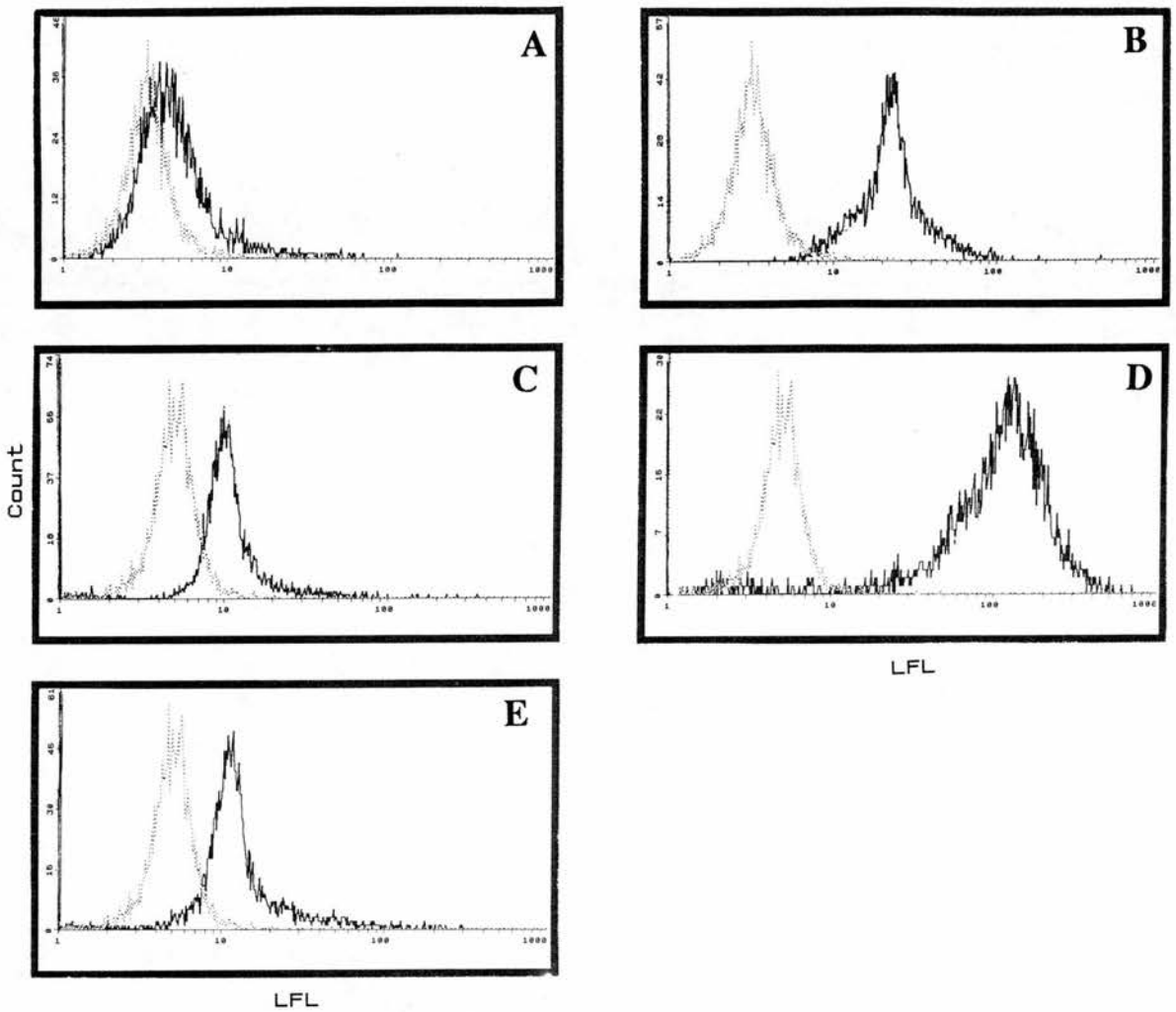


Figure 4.3
The Induction of Intracellular Respiratory Burst Activity by Pro-inflammatory Mediators.
Neutrophils were incubated with PAF (1 μM, 10 min, A and B), TNFα (200 U/ml, C and D), or buffer (30 min, E), in the presence of 1 μM DHR, and then treated with fMLP (100 nM, 10 min, B, D and E) or buffer (A and C). Samples were analyzed by flow cytometry (black outlines) and plotted against control neutrophils incubated with DHR alone (light grey outlines) (x-axis: logarithmic scale green fluorescence (LFL); y-axis: relative cell number). Representative flow-cytometry (EPICS Profile II) histograms (n = 10 in duplicate).

4.2.1.3 PAF-Induced Neutrophil Shape Change

In vivo, escalating concentrations of pro-inflammatory mediators are believed to guide neutrophils along a chemotactic gradient, whilst directing a specific sequence of neutrophil effector responses. Thus, a mediator that can prime the respiratory burst may simultaneously elicit other important functional responses. However, since specific neutrophil functions are required at different times during the activation sequence, one would expect that certain responses are more sensitive than others to a given concentration of a pro-inflammatory agent (Baggiolini and Dewald, 1986). One of the earliest neutrophil responses initiated is a change of shape (polarization), a very sensitive indicator of chemokinetic or chemotactic activity (Haston and Shields, 1985) which has been shown to correlate with the degree of priming of respiratory burst activity (Haslett *et al.*, 1985). Therefore, the concentration-dependency of PAF-induced neutrophil shape change was assessed in parallel with the priming of fMLP-stimulated superoxide anion release.

A 10 min incubation with ≤10 nM PAF had only a minimal effect on resting neutrophil morphology but, thereafter, PAF elicited a concentration-dependent increase in neutrophil shape change (EC_{50} 110 ± 27 nM) that was again maximal with 1 μM PAF (Figure 4.4a). At a higher concentration of 10 μM PAF, there was less deviation in the mean forward light scatter of neutrophils (assessed by flow cytometry) than with 1 μM PAF: this observation agrees with a previous report showing that the maximal chemotactic migration of neutrophils into cellulose filters occurred with 1 μM PAF and was reduced at higher concentrations (Shaw *et al.*, 1981). However, the apparent reduction in neutrophil shape change seen with 10 μM PAF correlated with the light-microscopic observation of large, round, "glassy"-looking neutrophils, an appearance suggestive of cell swelling. A further increase in the concentration to 100 μM PAF caused a 50% reduction in neutrophil viability (assessed by trypan blue extrusion). This agrees with the previous reports that >1 μM PAF can disrupt both membrane bilayers and whole cells (Sawyer and Andersen,

1989; Hoffman *et al.*, 1984), possibly due to the detergent-like properties of micelles that form at these concentrations (Kramp *et al.*, 1984).

Numerous reports have suggested that PAF may bind to carrier proteins in the circulation, especially albumin. These include the demonstration that when exogenous PAF is added to plasma it co-elutes with albumin and other plasma proteins (Ludwig *et al.*, 1985), and that PAF release from neutrophils is dependent upon the presence of extracellular albumin (Bratton *et al.*, 1991; Ludwig *et al.*, 1985). Albumin can also inhibit the binding of PAF to human neutrophil membranes (Valone, 1987), and may thus compete with cells and cell membranes for PAF. The current theory therefore predicts that albumin and other plasma proteins bind to PAF that is released from cells, and may even "extract" PAF and related molecules from the outer leaflet of cell membranes (Bratton *et al.*, 1994). In view of the potential role of albumin *in vivo*, its influence upon PAF-induced neutrophil shape change was examined. Human albumin (1.25 μg/ml) was found to have no significant effect upon the concentration-dependent increase in neutrophil shape change induced by PAF (Figure 4.4b). In view of these findings, a concentration of 1 μM PAF, without albumin present, was used for all further investigations of PAF-induced neutrophil responses.

The degree of neutrophil shape change elicited by PAF was compared to that induced by other chemotactic agents. Thus, PAF (1 μM, 10 min), TNFα (200 U/ml, 30 min) and fMLP (100 nM, 10 min) elicited 48 ± 8%, 68 ± 7% and 76 ± 5% shape change, respectively (Figure 4.5). When PAF and TNFα-treated neutrophils were subsequently stimulated with fMLP (100 nM, 10 min), there was a further 33 + 6% and 14 ± 5% increase in shape change, respectively.

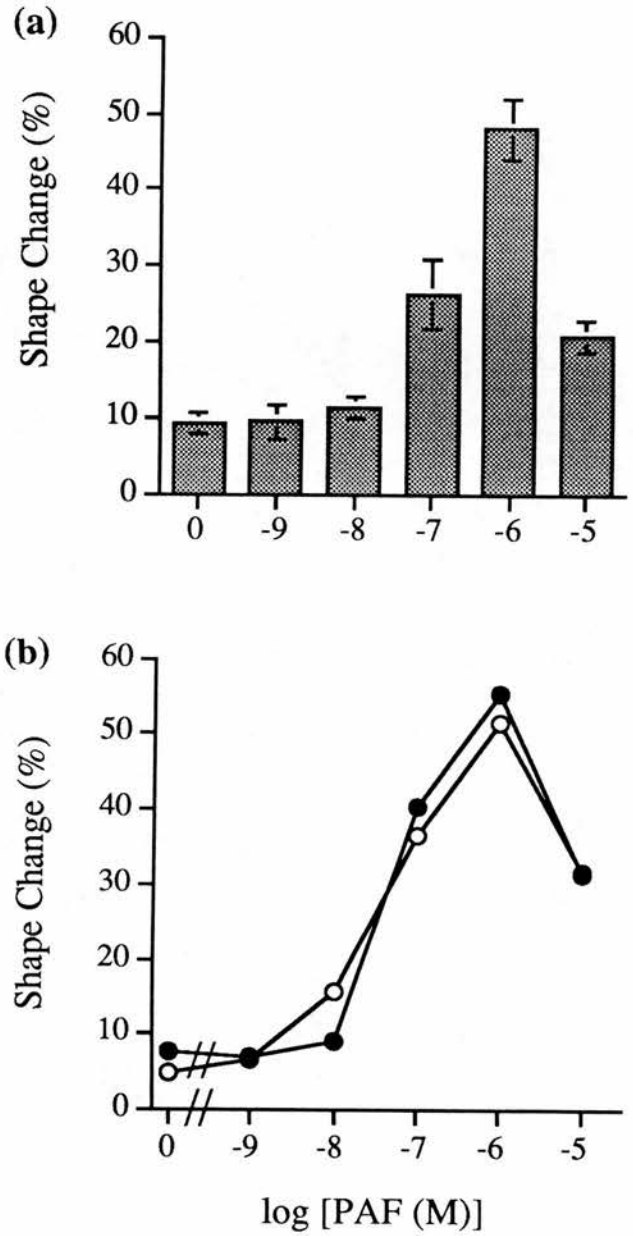


Figure 4.4
Concentration-Response for PAF-Induced Neutrophil Shape Change.
(a) Concentration-response data for PAF-induced shape change. Neutrophils were incubated with PAF (10 nM-10 μM, 10 min) or buffer control, then analyzed for percent shape change by flow cytometry (mean ± SEM, n = 6 in duplicate). (b) Effect of albumin on PAF-induced shape change. Neutrophils were treated as in (a) in the presence (open symbols) or absence (closed symbols) of 1.25 μg/ml human albumin (mean of triplicate determination from representative experiment of 3).

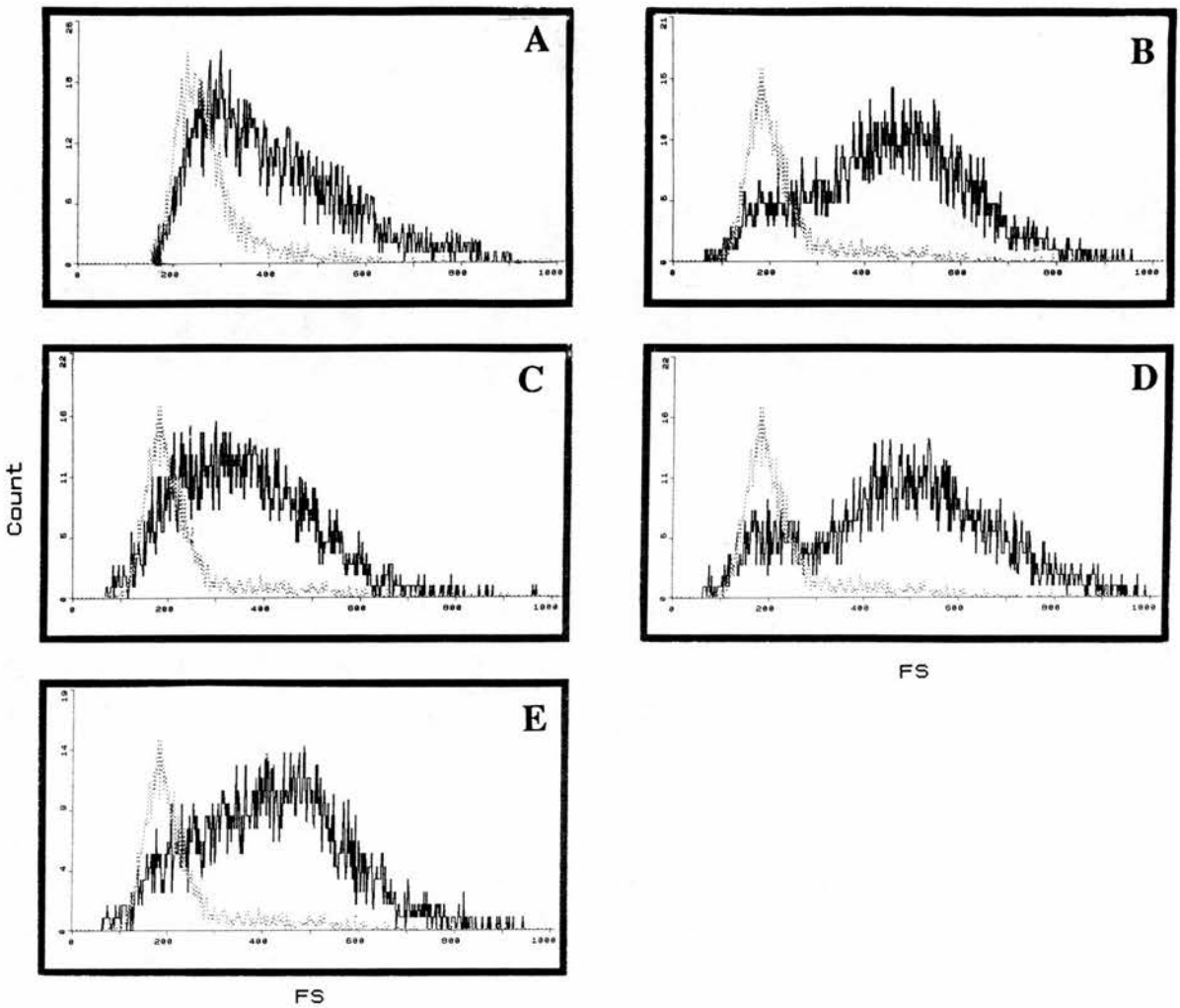


Figure 4.5
The Induction of Neutrophil Shape Change by Pro-inflammatory Mediators.
Neutrophils were incubated with PAF (1 μM, 10 min, A and B), TNFα (200 U/ml, 30 min, C and D), or buffer control (30 min, E), prior to treatment with fMLP (100 nM, 10 min, B, D and E) or buffer (A and C). Samples (black outlines) were analyzed for percent shape change by flow cytometry and plotted against control samples (light grey outlines) (x-axis: mean forward light scatter, FS; y-axis: relative cell number). Representative flow-cytometry (EPICS Profile II) histograms (n = 12 in duplicate).

4.2.1.4 Functional Upregulation of Adhesion Molecules

Although it has been demonstrated that neutrophil priming agents can increase the surface expression of CD11b/18, it is the functional upregulation of this molecule that is a more predictable indicator of neutrophil priming (Condliffe *et al.*, 1996). Therefore, PAF (1 μM, 10 min) and TNFα (200 U/ml, 30 min) were assessed for their ability to upregulate the binding capacity of CD11b/CD18, as a further index of their relative priming potentials. When incubated with neutrophils under their optimal priming conditions, PAF and TNFα caused a 3.6- and 4.2-fold functional upregulation of CD11b/18, respectively, when compared to control samples (Figure 4.6).

4.2.2 The Reversible Priming Induced by PAF

Having established the optimal priming conditions for PAF and its priming potential in relation to other pro-inflammatory agents, the next stage was to investigate whether its priming effects in human neutrophils were reversible. Since there had been two previous suggestions that priming of the fMLP-stimulated superoxide response by PAF might diminish spontaneously over a prolonged period (Vercellotti *et al.*, 1988; Gay *et al.*, 1986), we commenced with a detailed time-course of the effects of PAF on each of the chosen indices of priming. Owing to the short neutrophil life-span and the difficulty of maintaining isolated neutrophils in a basally-unprimed state, we restricted the length of the PAF preincubation period to 120 min.

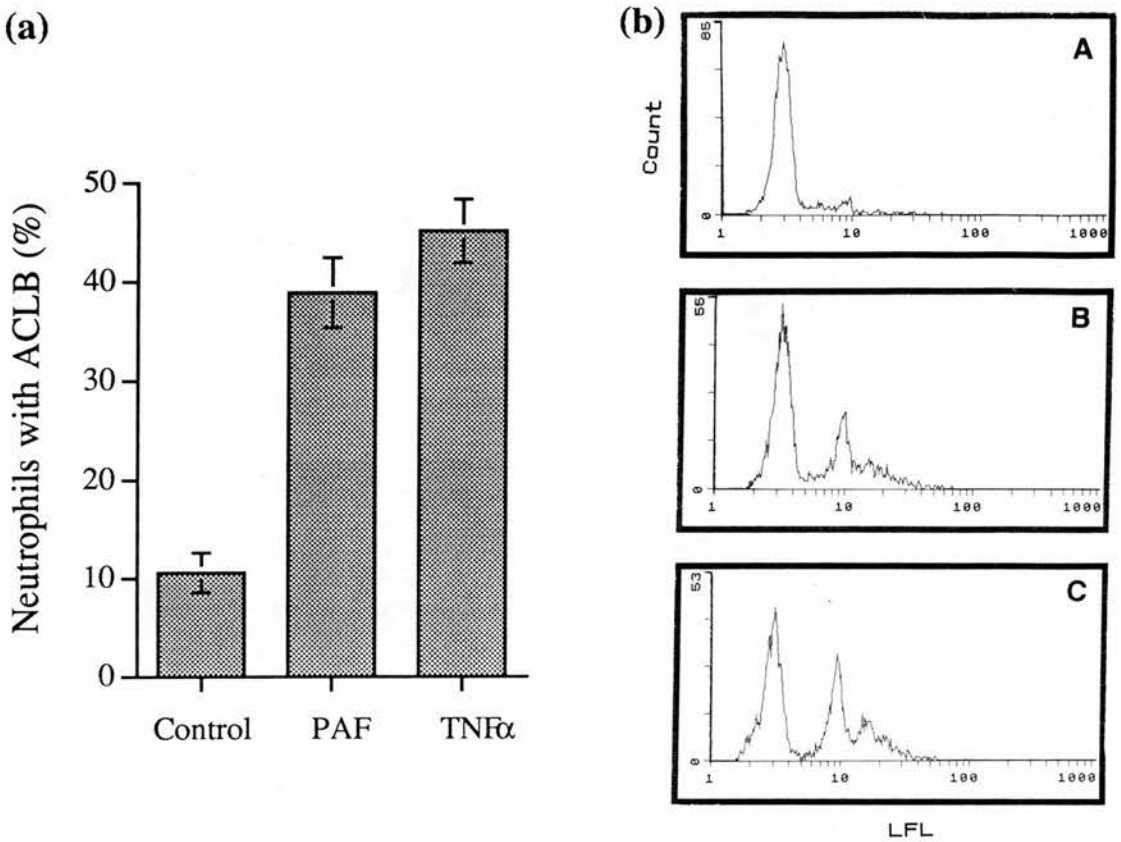


Figure 4.6

PAF- and TNFα-Induced Binding of ACLB in Human Neutrophils.

Neutrophils were incubated with PAF (1 μM, 10 min), TNFα (200 U/ml, 30 min), or buffer (30 min) in the presence of ACLB (0.75% v/v). Samples were analyzed by flow cytometry and the percentage of neutrophils with attached ACLB calculated by gating out the far left peak determined from control samples. (a) Mean ± SEM, n = 4 in duplicate. (b) Representative flow-cytometry (EPICS Profile II) histograms of control neutrophils (A) and neutrophils incubated with PAF (B) or TNFα (C) (x-axis: logarithmic scale green fluorescence, LFL; y-axis: relative cell number).

4.2.2.1 The Priming of the fMLP-Superoxide Response by PAF is Reversible

The prolonged incubation procedure itself did not initiate neutrophil priming, since cells at the end of the assay had a similar fMLP-superoxide response to those at the start (Figure 4.7). However, the ability of PAF to enhance the subsequent release of superoxide anions to fMLP was dependent upon the duration of the initial PAF pre-incubation. The maximal priming effect of 1 μM PAF occurred with a 10 min pre-incubation period; thereafter the enhancement of fMLP-stimulated superoxide anion release decayed so that by 2 hours the priming effect had completely disappeared, and when these "de-primed" neutrophils were stimulated with fMLP, the amount of superoxide they released was equivalent to that observed in cells that had never been primed. This spontaneous reversal of the priming effect of PAF displayed a consistently biphasic pattern, with an initial rapid loss in priming ($T_{½}$ 22 min) occurring after 10 min PAF exposure, followed by a second slower phase of decay ($T_{½}$ 34 min) that began after 40 min.

4.2.2.2 The Shape Change Response Induced by PAF is Reversible

Although neutrophils were seen to revert to an unprimed state following PAF-mediated priming of the fMLP-superoxide response, this recovery could have represented the down-regulation of a single pathway specific for this response. Therefore, the stability of the shape change elicited by PAF was also examined over a 2 hour period. When neutrophils were incubated with 1 μM PAF they underwent a rapid change in shape that was maximal within 2 min of the PAF addition (Figure 4.8). This shape change response subsequently reversed and by 30 min the PAF-treated neutrophils had almost resumed the round morphology of resting neutrophils. Following this recovery, these neutrophils gradually underwent a second phase of cell polarization that paralleled the small changes seen in control cells and continued to the end of the 2 hour incubation period.

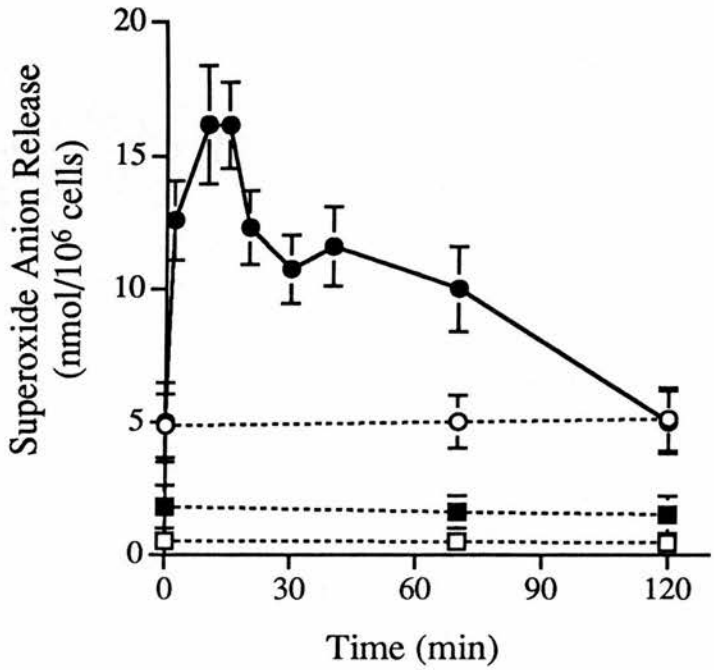


Figure 4.7
Time-Course for PAF-Induced Priming of fMLP-Stimulated Superoxide Anion Release in Human Neutrophils.
Neutrophils were incubated with PAF (1 μM, closed symbols) or buffer (open symbols) for 0-120 min, prior to treatment with fMLP (100 nM, 10 min, circles) or buffer control (squares) in the presence of 1 mg/ml cytochrome C. Reactions were terminated at the appropriate times by placing the cells on ice and superoxide anion release was assessed by scanning spectrophotometry (mean ± SEM, n = 4 in triplicate).

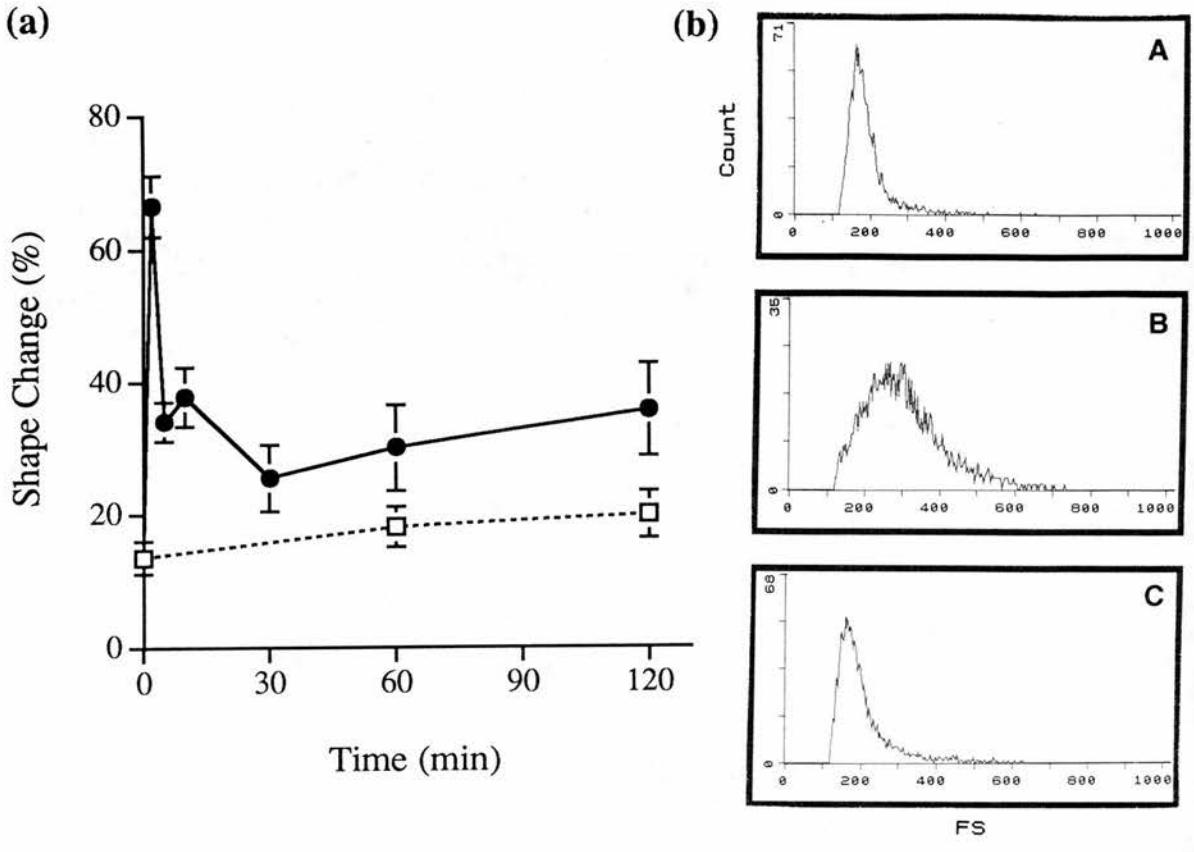


Figure 4.8
Time-Course for PAF-Induced Shape Change in Human Neutrophils.
(a) Time-course for PAF-induced shape change. Neutrophils were incubated with PAF (1 μM, closed circles) or buffer (open squares) for 0-120 min. Samples were analyzed for percent shape change by flow cytometry (mean ± SEM, n = 4 in duplicate). (b) Representative flow-cytometry (EPICS Profile II) histograms of control neutrophils (2 min, A) and neutrophils incubated with PAF for 2 min (B) or 30 min (C) (x-axis: mean forward light scatter, FS; y-axis: relative cell number).

4.2.2.3 The Functional Upregulation of CD11b/CD18 by PAF is Reversible

The demonstration that both the priming of the fMLP-superoxide response and the shape change induced by PAF are transient events might imply that neutrophil priming is a reversible process. However, if PAF was also shown to elicit a transient upregulation of the functional capacity of CD11b/CD18, this would represent a more global model of reversible neutrophil priming. The ability of 1 μM PAF to elicit a functional upregulation of CD11b/CD18 was measured as an increase in the binding of albumin-coated latex beads (ACLB) over a 2 hour incubation period: this effect was maximal after a 10 min incubation and then spontaneously declined to reach control levels by 2 hours (Figure 4.9).

4.2.3 Re-priming of De-primed Neutrophils

The above data demonstrate that PAF can elicit a transient and reversible enhancement of three different effector responses that are associated with neutrophil priming. However, for this priming to be considered truly reversible, a demonstration that these cells can be *re-primed* is also required. Thus, if sequentially primed and de-primed neutrophils had the innate capacity to be primed again without any significant loss in their priming potential, then a novel model of neutrophil activation status could be proposed, whereby neutrophils could cycle between the primed and quiescent state, as dictated by their inflammatory environment.

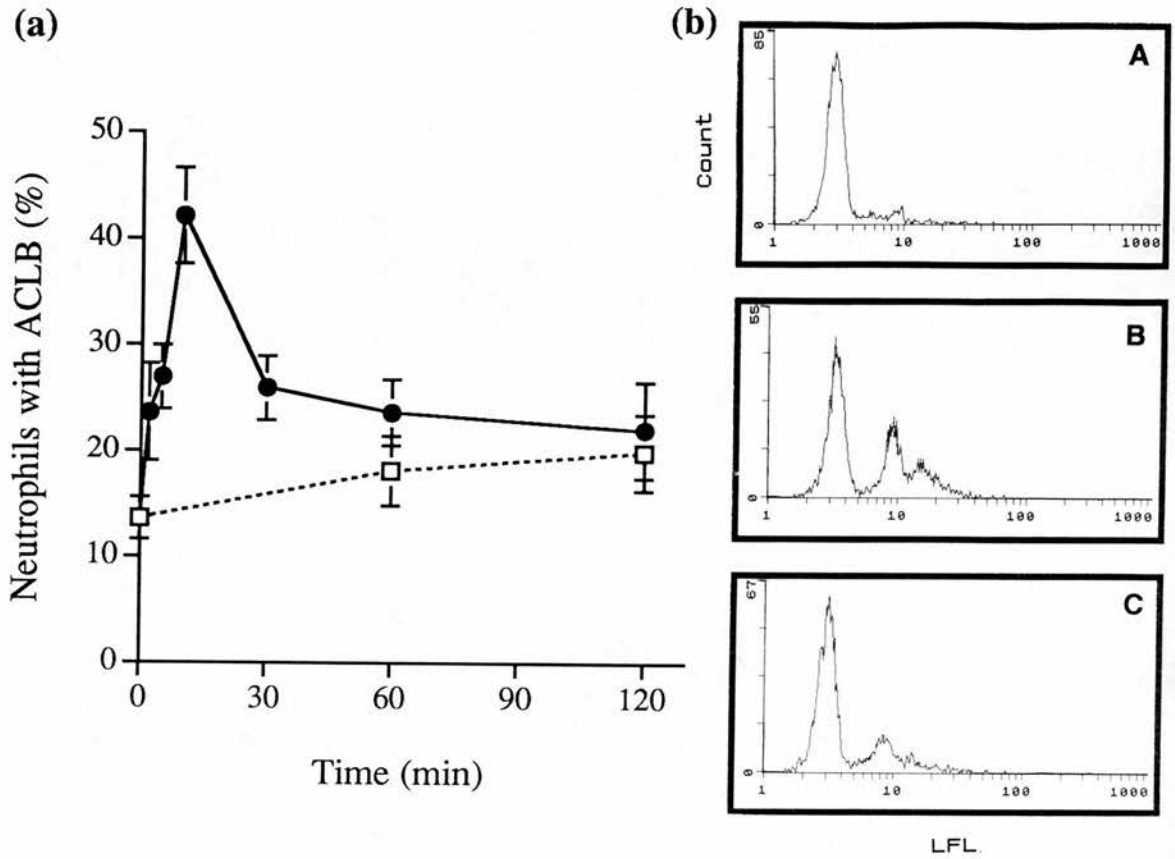


Figure 4.9
Time-Course for PAF-Induced Binding of ACLB in Human Neutrophils.
(a) Time-course for PAF-induced binding of ACLB. Neutrophils were incubated with PAF (1 μM, closed circles) or buffer (open squares) for 0-120 min. ACLB (0.75% v/v) were added 15 min before the termination of the reaction with 0.5% glutaraldehyde, except for time-points <15 min where beads were added before the agonist. Samples were analyzed for attached ACLB (against time-matched control samples) by flow cytometry (mean ± SEM, n = 4 in duplicate). (b) Representative flow-cytometry (EPICS Profile II) histograms of control neutrophils (10 min, A) and neutrophils incubated with PAF for 10 min (B) or 120 min (C) (x-axis; logarithmic scale green fluorescence, LFL; y-axis: relative cell number).

4.2.3.1 Re-Priming of Neutrophils with PAF and TNFα

Since it was previously shown that a prolonged 2 hour incubation procedure did not prime control neutrophils for an enhanced superoxide response to fMLP (Figure 4.7) but did elicit a small degree of shape change in these cells (Figure 4.8), we selected the cytochrome C reduction assay to address the more detailed and protracted investigations into the potential for neutrophils to be re-primed. When neutrophils that had primed and de-primed over a 2 hour incubation with PAF were challenged again, with either PAF (1 μM, 10 min) or TNFα (200 U/ml, 30 min), they retained their priming capacity, producing a similar superoxide anion response upon fMLP stimulation as freshly-primed cells (Figure 4.10).

4.2.3.2 Re-Priming after Hypotonic Shock

The re-priming potential of neutrophils that had recovered after a hypotonic challenge (see Chapter 3) was also investigated. PAF (1 μM, 10 min), by itself, elicited minimal superoxide anion release from neutrophils that had been incubated under isotonic conditions, but primed the fMLP response by 7.6-fold. Under hypotonic conditions, PAF further augmented the basally-primed fMLP response by 3.0-fold (Figure 4.11). In incubations where isotonicity was restored after the period of hypotonic priming, the resulting de-primed neutrophils could be re-primed by a subsequent exposure to PAF, albeit to a lesser degree (3.9-fold) than that observed in isotonically-maintained neutrophils. This reduction in priming potential was not a reflection of cell necrosis, as neutrophil viability was routinely >99% for all conditions studied. Thus, like neutrophils that had de-primed following a prolonged exposure to PAF, osmotically primed and de-primed neutrophils retained their capacity to be primed for a second time by a physiological agent such as PAF.

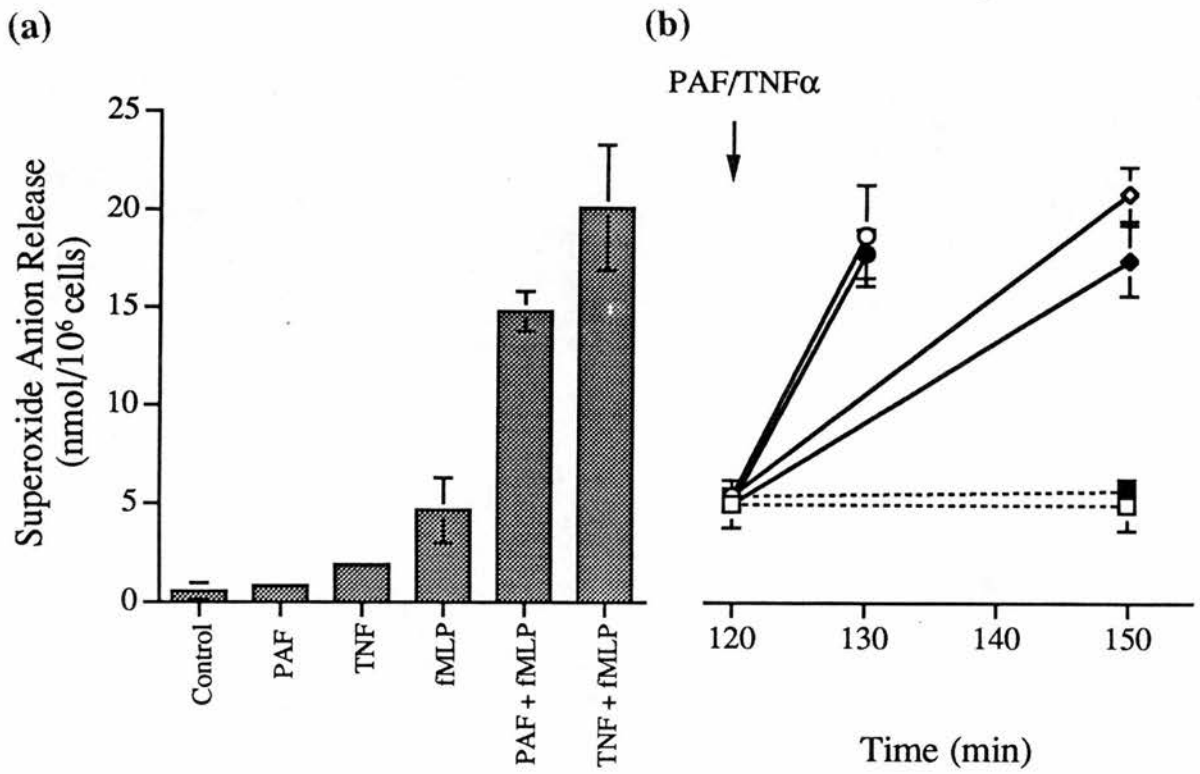


Figure 4.10

Re-priming of Human Neutrophils with PAF or TNFα Following Initial Priming with PAF.

(a) Superoxide anion priming with PAF and TNFα in freshly-isolated human neutrophils. Neutrophils were incubated with buffer, PAF (1 μM, 10 min) or TNFα (200 U/ml, 30 min), as these represent optimal priming conditions for later comparisons with re-primed neutrophils (see (b)). Following subsequent treatment with fMLP (100 nM, 10 min) or buffer in the presence of 1 mg/ml cytochrome C, superoxide anion release was assessed spectrophotometrically. (b) Superoxide anion re-priming of neutrophils with PAF and TNFα following a 120 min incubation with PAF. Neutrophils were incubated for 120 min with PAF (1 μM, closed symbols) or buffer (open symbols), followed by a second treatment with PAF (1 μM, 10 min, circles), TNFα (200 U/ml, 30 min, diamonds), or buffer (30 min, squares). Samples were then incubated with fMLP (100 nM, 10 min) and assessed for superoxide anion release as above (mean ± SEM, n = 3 in triplicate. Where not shown S.E.M. values fall within symbols).

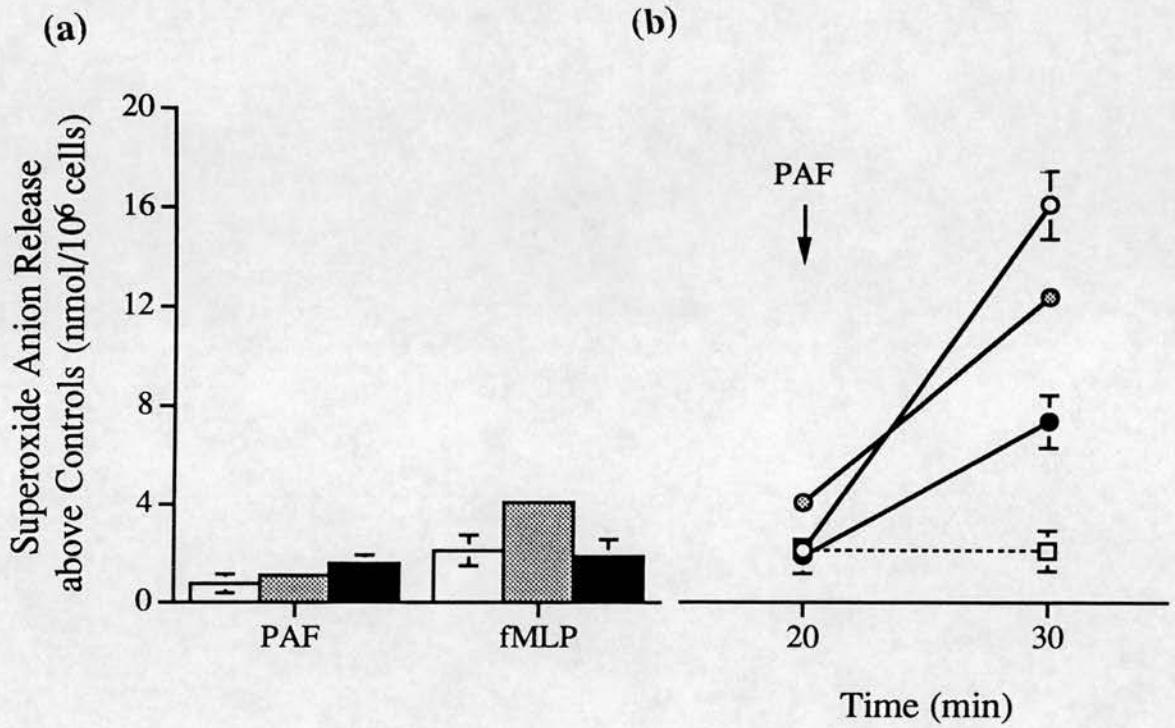


Figure 4.11
Re-priming of Human Neutrophils with PAF Following Initial Priming with Hypotonic Challenge.
(a) Superoxide anion priming of freshly-isolated human neutrophils with a hypotonic challenge. Neutrophils were incubated for 19 min in isotonic PBS (150 mM NaCl, white bars) or hypotonic PBS (50 mM NaCl, grey and black bars), prior to 1 min treatment with 5M NaCl (to reverse hypotonicity to isotonicity, black bars) or PBS (to retain tonicity, white and grey bars). Following incubation with fMLP (100 nM, 10 min) or buffer in the presence of 1 mg/ml cytochrome C, superoxide anion release was assessed spectrophotometrically. (b) Superoxide anion re-priming of neutrophils with PAF following a hypotonic challenge. Neutrophils were incubated in continuously isotonic (white symbols), continuously hypotonic (grey symbols), or hypotonic then isotonic conditions (black symbols) as detailed above for 20 min. This was followed by treatment with PAF (1 μM, 10 min, circles) or isotonic buffer (10 min, squares), and subsequent incubation with fMLP (100 nM, 10 min). Samples were assessed for superoxide anion release as above (mean ± SEM, n = 3 in triplicate).

4.3 Discussion

The neutrophil can exist in a number of different functional states, and this has a significant bearing on its behaviour and responsiveness *in vitro*. Thus, in the unprimed state, the neutrophil displays little or no secretory response when incubated with an agent such as fMLP, whereas such a challenge in a fully primed cell results in an explosive increase in the rate of respiratory burst activity (Gay *et al.*, 1986; Pinckard *et al.*, 1992); this priming-activation axis has been shown to be a major determinant of neutrophil behaviour *in vivo* (Warren *et al.*, 1989). However, the very protracted priming effects of certain pro-inflammatory mediators such as LPS, G-CSF, GM-CSF and IFN-γ, together with the short neutrophil life-span, has implied that priming by these agents is a largely irreversible process (Roberts *et al.*, 1993; Guthrie *et al.*, 1984; Carey *et al.*, 1994; Ichinose *et al.*, 1990). Indeed, the sustained nature of the neutrophil priming effect has been postulated to play a fundamental role in the long-term inflammatory response observed with certain agents, including endotoxin (Carey *et al.*, 1994).

However, on detailed examination of the published literature, it became apparent that neutrophil priming might, under certain circumstances, be a transient process. The most convincing example of this was the reversible priming of the fMLP-superoxide response induced by hypotonic shock (Edashige *et al.*, 1993), which we confirmed in Chapter 3. This priming may have been a result of the reversible neutrophil swelling that occurs under hypotonic conditions (Miyahara *et al.*, 1993), with its associated disordering of plasma membrane-cytoskeletal interactions and net increase in cell-surface negative charge. Similar membrane perturbations might also explain the transient, receptor-independent, priming effects of $InsP_6$ that were observed in Chapter 3. However, if these afore-mentioned studies were collectively considered as physico-chemical manipulations, the remaining literature provided little direct information about whether established, receptor-mediated priming agents could reversibly enhance neutrophil responses.

In this Chapter, we have provided evidence that the neutrophil priming effects of an important pro-inflammatory mediator, PAF, are transient. Since the spontaneous de-priming of neutrophils observed following PAF treatment was apparent for fMLP-stimulated superoxide anion generation, CD11b function, and cell polarization, it was unlikely to represent the selective down-regulation of any one particular component of the priming response. We have also demonstrated that these PAF-primed-de-primed neutrophils can go through a further complete cycle of priming (by either TNFα or PAF) and activation, with the maintenance of full viability throughout. Thus, the spontaneous decay of the PAF priming effect was not merely a consequence of the extended incubation procedure affecting cell integrity or metabolic status, and was unlikely to reflect the down-regulation of receptor number or affinity.

Following a 60 min pre-incubation with 1 μM PAF, the priming of the fMLP-superoxide response was reduced to approximately 50% of its maximal value, in line with a previous observation (Gay *et al.*, 1986), and had completely disappeared within 2 hours. However, the spontaneous reversal of this priming effect exhibited a consistently biphasic pattern of decay, suggesting that two different, but interacting, mechanisms might be responsible for the de-priming process. Furthermore, a two-stage reversal of the primed response has been observed with 10 nM PAF, although this effect was more rapid, being complete within 60 min (Vercellotti *et al.*, 1988). The apparent discrepancy in the rates of reversal may be a consequence of the different concentrations of PAF used in these studies. Thus, if lower concentrations of PAF elicit more transient priming responses, this implies that the rate of de-priming might, in part, reflect: (i) the degree of PAF receptor occupancy and intensity of the priming signal; and (ii) the rate of removal of PAF (e.g. by metabolism or internalization) from the incubation medium.

However, a novel model has recently been proposed for PAF-induced priming of superoxide responses (Pinckard and Prihoda, 1996). It was reported that PAF could

elicit two different types of neutrophil priming: at low concentrations (10 pM-1 nM) PAF induced a small priming effect that remained stable over time, whilst the much greater priming effects of higher concentrations of PAF (1-100 nM) were more transient. For example, the maximal enhancement of fMLP-stimulated superoxide anion release by 10 nM PAF was reduced by approximately 25% within 10 min of the PAF addition (Pinckard and Prihoda, 1996), an observation similar to that previously reported (Vercellotti *et al.,* 1988). Furthermore, reversal of the PAF (1-100 nM)-primed C5a-induced superoxide response was even more dramatic, being complete within 10 min (Pinckard and Prihoda, 1996). Thus, it was suggested that there may be at least two effector pathways, mediated by high- and lower-affinity PAF receptors, that modulate PAF-induced priming of respiratory burst activity in human neutrophils. According to this model, the reversible priming we observed with 1 μM PAF would largely be mediated by a subtype or conformational state of the PAF receptor that was of relatively low-affinity.

As the second index of neutrophil priming, PAF-induced shape change occurred rapidly, being maximal within 2 min of PAF (1 μM) addition. This response was also transient and by 30 min had largely reversed, supporting the hypothesis that PAF induced neutrophil priming might be a reversible phenomenon. In contrast, both C5a and fMLP have been reported to elicit a rapid polarization response in neutrophils that is sustained for at least 1 hour (Haston and Shields, 1985; Smith *et al.,* 1979); however, upon removal of the stimuli, this shape change subsequently reversed (Smith *et al.,* 1979). Taken together, these observations imply that neutrophil shape change will be maintained if the chemotactic factor is continuously present, but may reverse when neutrophils are no longer stimulated. Thus, the spontaneous reversal of PAF-elicited shape change may be due to a "removal" of PAF from the incubation medium during the 2 hour period.

The delayed, gradual increase in shape change that occurred when neutrophils were incubated for >30 min with PAF was unlike the initial PAF-mediated response in that it: (i) was smaller and much slower to evolve; (ii) paralleled changes seen in control

neutrophils; and (iii) was not accompanied by a similar gradual increase in the functional upregulation of CD11b/CD18 or priming of the superoxide response to fMLP (although it may have contributed to its biphasic pattern of reversal). Therefore, it is unlikely that this delayed neutrophil shape change was mediated directly by PAF. However, it could have reflected an event that was secondary to prolonged neutrophil incubation, for example the synthesis and/or release of an autocrine/paracrine neutrophil chemotactic agent.

PAF also upregulated the functional capacity of CD11b/CD18, with a similar time-course to that observed for the priming of superoxide anion release. This transient effect has recently been confirmed with 100 nM PAF (Condliffe *et al.*, 1996). However, in this latter report it was also demonstrated that the accompanying upregulation of CD11b expression (in contrast to function) was not reversible, but was maintained at high levels for at least 2 hours. Furthermore, since lower concentrations of PAF were required to upregulate the functional capacity, as opposed to the surface expression, of CD11b/CD18, it was suggested that CD11b/CD18 function (measured by the binding of ACLB) might be the more sensitive indicator of neutrophil priming (Condliffe *et al.*, 1996). Therefore in retrospect, the small yet transient enhancement of respiratory burst activity elicited by IL-8 (Daniels *et al.*, 1992; Roberts *et al.*, 1993) may have been another example of reversible neutrophil priming, even though the increased surface expression of CD11b was sustained; the patho-physiological significance of this event, however, was entirely overlooked by these authors.

This chapter has focused solely on the *in vitro* effects of soluble PAF, which may mimic the *in vivo* paracrine action of PAF (possibly bound to serum albumin) that has been released from inflammatory cells. However, PAF may also be present in a bound form within the vasculature when it is co-expressed with P-selectin on the surface of activated endothelial cells, and thereby mediates a juxtacrine effect on rolling (and thus momentarily immobilized) neutrophils (Lorant *et al.*, 1993; Lorant *et al.*, 1991). PAF is expressed only transiently on the endothelial surface, being

maximally up-regulated within 10 min (Prescott *et al.*, 1984; McIntyre *et al.*, 1985), and is then rapidly degraded (McIntyre *et al.*, 1986; McIntyre *et al.*, 1985). This reversible expression of PAF has been shown to parallel both the transient adhesion of neutrophils to the activated endothelial surface (Lorant *et al.*, 1991) and the priming of fMLP-stimulated superoxide anion release (Vercellotti *et al.*, 1989), responses that are facilitated by P-selectin (Lorant *et al.*, 1993). Thus, it has been postulated that the reversible endothelial co-expression of a tethering (P-selectin) and a signalling (PAF) molecule may provide a strictly-controlled mechanism for the efficient adhesion and functional upregulation of neutrophils at their first committed step of an acute inflammatory response (Lorant *et al.*, 1991).

It has subsequently been demonstrated that the majority of "PAF" synthesized by vascular endothelial cells is actually not the classical *alkyl*-PAF but *acyl*-PAF (Whatley *et al.*, 1992; Triggiani *et al.*, 1991). Thus, it may be acyl-PAF that mediates the functional upregulation of neutrophils that are rolling along the endothelium. Acyl-PAF in suspension has been shown to prime human neutrophils for an enhanced superoxide anion response to fMLP and C5a but, in contrast to alkyl-PAF, cannot elicit chemotaxis (Pinckard *et al.*, 1992). Furthermore, the priming induced by acyl-PAF is slower to evolve and more sustained than the priming induced by fluid-phase alkyl-PAF (Pinckard and Prihoda, 1996). However, since certain bioassays for PAF cannot adequately distinguish between alkyl- and acyl-PAF (Mueller *et al.*, 1991; Bratton *et al.*, 1994), it may be that previous investigations with endothelial-cell associated "PAF" were actually performed with acyl-PAF. If this was the case, then the apparently different priming characteristics of endothelial cell-associated- and fluid-phase- acyl-PAF may be due to the modulatory effects of P-selectin.

In view of the above observations, it would appear that there may be at least two distinct types of PAF-induced neutrophil priming *in vivo*: (i) alkyl-PAF released from activated inflammatory cells may bind to serum albumin and prime neutrophils in suspension; and (ii) acyl-PAF expressed on the surface of activated endothelial

cells may prime rolling neutrophils that have been temporarily immobilized by P-selectin. In addition, the reversible nature of the responses induced by both fluid-phase and endothelial cell-associated PAF may limit the effects of this potent pro-inflammatory mediator. Since the transient expression of PAF by activated endothelial cells is believed to explain the resulting transient neutrophil responses (Lorant *et al.,* 1991), a similar short-lived appearance of PAF in the incubation medium might explain the reversible priming that we observed with suspended neutrophils. Thus, if this idea is applicable to the *in vivo* situation, then the PAF released from activated inflammatory cells may remain only briefly in the circulation and thereby limit the pleiotropic effects of PAF to the initial phase of an acute inflammatory response.

The above hypothesis is supported by reports indicating a central role for endothelial cell-associated PAF in the priming (Hill *et al.,* 1994) and extravasation (Nourshargh *et al.,* 1995) of neutrophils exposed to IL-1β-activated endothelium. Thus, any delay in neutrophil exit through an activated endothelial surface might permit cell recovery and the return of un-primed neutrophils to the circulation. The recognition that neutrophils have the potential to de-prime may therefore provide an additional point of control in the earliest stage of an acute inflammatory response, whereby cells may return to their former quiescent state and potentially re-join the circulating neutrophil pool. These de-primed neutrophils, once fully recovered, could again attain their maximal priming potential and mount subsequent inflammatory responses as dictated by ensuing inflammatory challenges. However, in the wake of a more widespread or prolonged inflammatory insult, it is unlikely that neutrophils would be exposed solely to PAF or other transient priming agents, and the presence of agents such as LPS, GM-CSF and G-CSF might promote a synergistic mechanism of priming to maintain the prolonged up-regulation of neutrophil responses. Nevertheless, the recognition of reversible neutrophil priming may provide a novel target for counteracting the pro-inflammatory, and potentially tissue-damaging, effects of primed and fully activated neutrophils.

5. CHAPTER 5: POTENTIAL MECHANISMS UNDERLYING THE REVERSIBLE PRIMING INDUCED BY PLATELET-ACTIVATING FACTOR

5.1 Introduction

Having demonstrated in Chapter 4 that PAF can induce transient priming of human neutrophils, the reasons for this reversibility were next considered. Initially, it was important to exclude any effects that were merely a consequence of the prolonged incubation procedure, for example, the decline of either reagent or neutrophil activity. Following this, several potential mechanisms were targeted, including: (i) receptor-dependent events; (ii) the tyrosine phosphorylation of intracellular proteins; (iii) the metabolism of PAF; and (iv) modulatory effects of other inflammatory agents.

There is a general consensus that neutrophils contain specific, high-affinity PAF receptors that transmit the bioactions of PAF and are inhibitable by a wide variety of PAF receptor antagonists (Casals-Stenzel *et al.*, 1987; Shen *et al.*, 1985; O'Flaherty *et al.*, 1989; Dent *et al.*, 1989; Marquis *et al.*, 1988; Hwang, 1988). A down-regulation of either the number or functional capacity (i.e. coupling) of neutrophil PAF receptors could underlie the reversal of PAF-mediated priming. Thus, neutrophil de-priming may be similar to the homologous desensitization that has previously been observed when cells become unresponsive to PAF following repeated exposure (Schwertschlag and Whorton, 1988; O'Flaherty *et al.*, 1981; Henson, 1976; Benveniste *et al.*, 1972). Several mechanisms have been proposed to mediate this process, including: receptor down-regulation (Sibley *et al.*, 1987; O'Flaherty *et al.*, 1992; Scarpace and Abrass, 1982); direct inactivation of G-proteins (OFlaherty *et al.*, 1992; Milligan and Green, 1991); or the modulation of intracellular signal transduction pathways (Galizzi *et al.*, 1987). Since these same, receptor-

dependent mechanisms may apply to the reversal of PAF-mediated priming, we initially investigated the contribution of PAF receptor occupancy using the PAF receptor antagonists, WEB 2086 and UK-74,505.

Recently, there has been growing interest in a group of intracellular proteins that become tyrosine-phosphorylated upon neutrophil priming and/or activation. Virtually all of the known neutrophil stimulants, including PAF (Nick *et al.*, 1997; Gomez-Cambronero *et al.*, 1991), TNFα (Lloyds *et al.*, 1995; Akimaru *et al.*, 1992) and fMLP (Nick *et al.*, 1997; Ohta *et al.*, 1992), have been shown to elicit these events in a concentration- and time-dependent manner, often producing a very similar pattern of tyrosine phosphorylation (Richard *et al.*, 1994). Furthermore, tyrosine phosphorylation of certain proteins has been linked to diverse functional responses of neutrophils, including priming (Lloyds and Hallett, 1994; Kanbara *et al.*, 1993; Akimaru *et al.*, 1992; Lloyds *et al.*, 1995) and, in the case of hypotonic shock, to reversible priming (Edashige *et al.*, 1993). This intracellular signalling mechanism is reminiscent of that used by growth factors, which act through receptors with intrinsic tyrosine kinase activity (Cadena and Gill, 1992). Although the majority of neutrophil stimulants signal through receptors which do not possess an intrinsic tyrosine kinase domain, neutrophils do contain tyrosine-specific protein kinases (Kraft and Berkow, 1987; Huang *et al.*, 1988) and phosphatases (Fialkow *et al.*, 1994; Kansha *et al.*, 1993). Thus, these enzymes may mediate the tyrosine phosphorylation, and subsequent de-phosphorylation, of intracellular proteins as part of a rapid and reversible signalling pathway for neutrophil stimulation.

A gradual decline in the bioactivity of PAF during the assay might also explain the transient priming effects of PAF. For instance, if PAF was degraded extracellularly, or internalized by neutrophils and subsequently metabolized, one would predict that the actual concentration of PAF would decrease over the incubation period and perhaps fall below a critical threshold for neutrophil priming (approximately 1-10 nM, Chapter 4). It is known, for example, that PAF can be degraded by the action of PAF acetylhydrolases, a group of enzymes which remove the acetyl group at the *sn*-2

position of the PAF molecule to yield the biologically-inactive products, lyso-PAF and acetate (Figure 5.1). These enzymes regulate the levels of PAF in the plasma (Pinckard *et al.*, 1979; Farr *et al.*, 1980) and in tissues (Alam *et al.*, 1983; Blank *et al.*, 1981): human neutrophils have been shown to inactivate PAF in their plasma membranes at a rate of 1 nmol PAF per 10^7 neutrophils per minute (O'Flaherty *et al.*, 1986). However, if an *N*-methylcarbamyl residue is introduced at the *sn*-2 acetyl position of PAF, this yields a biologically-active PAF analogue, C-PAF (Figure 5.1), which is completely resistant to metabolic inactivation by human neutrophils or serum (Tessner *et al.*, 1989; O'Flaherty *et al.*, 1987). Thus, we used C-PAF to investigate whether the metabolism of PAF played a significant role in the reversal of PAF-mediated priming.

Many inflammatory agents have been reported to modulate the responses of stimulated neutrophils. These agents include: (i) prostaglandins, such as PGE_2 (Rossi and O'Flaherty, 1989); (ii) leukotrienes, such as LTB_4 (Ford-Hutchinson *et al.*, 1980; Lin *et al.*, 1982); and (iii) adenine nucleotides, such as ATP, ADP, AMP, and adenosine (Ward *et al.*, 1988; McGarrity *et al.*, 1989). Some of these agents may be released upon neutrophil stimulation to act as autocrine/paracrine regulators of neutrophil function. For example, activated human neutrophils have been shown to synthesize and release LTB_4 (Hopkins *et al.*, 1983), a potent neutrophil chemoattractant (Ford-Hutchinson *et al.*, 1980) and inducer of shape change (Rossi *et al.*, 1993), but a relatively weak priming agent for superoxide anion release (Baggiolini and Dewald, 1986). Thus, it is possible that the release of such an agent might underlie the secondary, delayed increase in neutrophil shape change that occurred following the reversal of PAF-mediated shape change. However, in order to mediate the spontaneous reversal of neutrophil priming, the agent in question would also have to inhibit the early PAF-induced responses.

Adenosine acts through specific, cell-surface receptors to inhibit many neutrophil functions, especially those elicited by fMLP or C5a. For example, micromolar concentrations of adenosine have been shown to inhibit: neutrophil rolling (Asako *et*

al., 1993) and adhesion to endothelial cells (Cronstein *et al.,* 1992); the generation of reactive oxygen species, including superoxide anions (Stewart and Harris, 1993; Walker *et al.,* 1990; Ward *et al.,* 1988; Cronstein *et al.,* 1983) and H_2O_2 (Cronstein *et al.,* 1987), possibly by the uncoupling of G-proteins from fMLP receptors (Burkey and Webster, 1993); and neutrophil-mediated injury to endothelial cells (Cronstein *et al.,* 1986). Furthermore, adenosine has been reported to completely inhibit PAF (1 μM)-induced priming of fMLP-stimulated superoxide anion release in human neutrophils (Stewart and Harris, 1993). However, in contrast to these inhibitory actions, adenosine has also been reported to have no effect on (McGarrity *et al.,* 1989), or even promote (Rose *et al.,* 1988; Garcia-Castro *et al.,* 1983), neutrophil chemotactic responses to fMLP. Since adenosine is a ubiquitous product of normal cellular activity, its release from neutrophils (or any contaminating platelets) might mediate the paracrine modulation of neutrophil responsiveness. Thus, adenosine release was investigated as a potential explanation for both the early reversal of PAF-induced priming, and the delayed, secondary increase in neutrophil shape change.

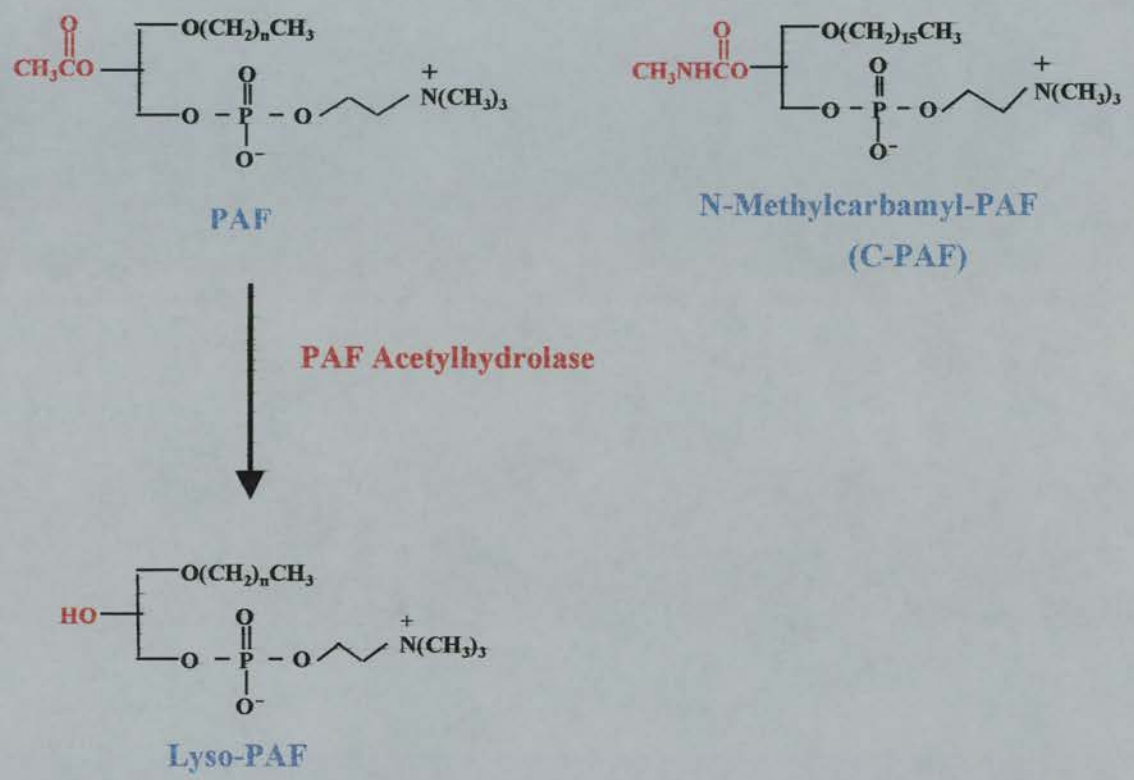


Figure 5.1
The Metabolism of PAF by PAF Acetylhydrolase.
PAF acetylhydrolase removes the *sn*-2 acetyl group (red) of PAF to yield the biologically-inactive products lyso-PAF and acetate. C-PAF contains an N-methylcarbamyl residue at the *sn*-2 position and is a non-metabolizable, yet biologically-active, analogue of PAF.

5.2 Results

5.2.1 Degradation of Cytochrome C

The possibility that neutrophil "de-priming" might reflect the degradation of cytochrome C during the 2 hour incubation could not be overlooked. Hence, its intra-assay stability was examined. A direct comparison of cytochrome C that had been incubating at 37°C for the whole 2 hour period, with cytochrome C that was prepared immediately before the 2 hour time-point, revealed no significant difference in the calculated superoxide responses to fMLP (nmol/10^6 neutrophils: 4.7 ± 0.4 (2 hour-old cytochrome C); 5.0 ± 0.3 (fresh); n = 3). Thus, the degradation of cytochrome C during the assay was not responsible for the reversal of PAF-mediated priming.

5.2.2 Altered Responsiveness of Neutrophils

It was considered possible that the priming potential of neutrophils may have altered during the prolonged incubation procedure, resulting in a diminished secretagogue response at the later time-points. Since neutrophil viability remained ≥99% throughout the 2 hour incubation period, and neutrophils could subsequently be re-primed without a significant reduction in their activation potential (Chapter 4), the reversal of PAF-primed responses was not secondary to a decline in cell viability. Nevertheless, it was still possible that the overall responsiveness of neutrophils could have fluctuated over the incubation period.

PMA is a phorbol ester that activates PKC directly and has been used widely in neutrophil signal transduction studies to evoke consistent and large respiratory burst responses (Daniels *et al.*, 1994; Smith and Weidemann, 1993; Keller *et al.*, 1995;

Majumdar *et al.*, 1991). This agent can therefore provide an indication of the overall responsiveness of a neutrophil population, as well as the intrinsic activity of PKC. Thus, neutrophils were stimulated with PMA (100 ng/ml, 60 min) at various times throughout the 2 hour incubation period and assessed for superoxide anion release and shape change (Figure 5.2). Both responses remained stable throughout, again implying that there was no significant loss in neutrophil responsiveness over the 2 hour incubation procedure.

5.2.3 Metabolism of PAF

Neutrophils can use PAF acetylhydrolase to rapidly inactivate PAF (O'Flaherty *et al.*, 1986). Therefore, as significant PAF metabolism may have occurred during the 2 hour incubation, the time-course for priming of fMLP-stimulated superoxide anion release by PAF was compared with that of its non-metabolizable analogue, C-PAF (Calbiochem, Nottingham, UK) (Figure 5.1). The time-courses were found to be almost identical (Figure 5.3). Thus, the spontaneous reversal of the PAF-primed superoxide response was not secondary to the intra-assay metabolism of PAF: however, a metabolically-independent reduction in the bioactivity of PAF (and C-PAF) could not be excluded.

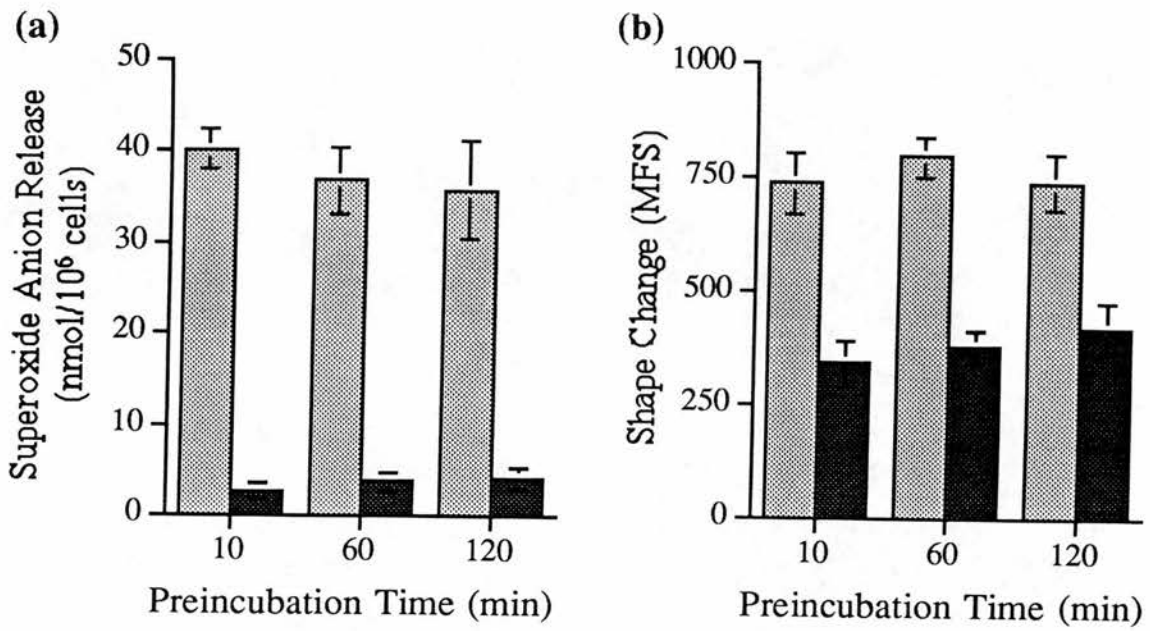


Figure 5.2
Responsiveness of Human Neutrophils to PMA.
(a) Superoxide anion response of human neutrophils to PMA. Neutrophils were incubated for 10-120 min, prior to stimulation with PMA (100 ng/ml, 60 min) or buffer, in the presence of 1 mg/ml cytochrome C. Superoxide anion release was assessed spectrophotometrically. (b) Shape change response of human neutrophils to PMA. Neutrophils were incubated as detailed in (a) in the absence of cytochrome C, and analyzed for percent shape change by flow cytometry (mean ± SEM, n = 3 in duplicate).

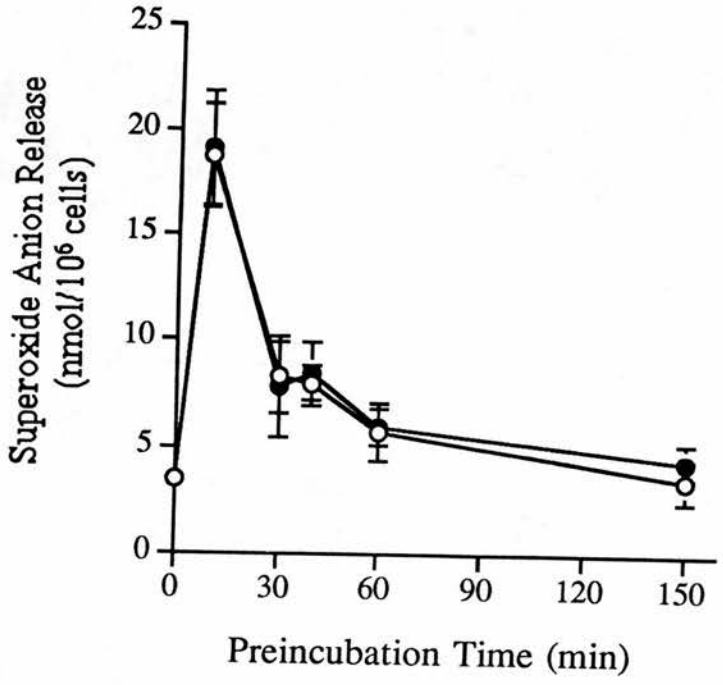


Figure 5.3
Time-Course for Priming of fMLP-Stimulated Superoxide Anion Release in Human Neutrophils by PAF and C-PAF.
Neutrophils were incubated with PAF (1 μM, closed symbols) or C-PAF (1 μM, open symbols) for 0-120 min, prior to treatment with fMLP (100 nM, 10 min) in the presence of 1 mg/ml cytochrome C. Reactions were terminated at the appropriate times by placing the cells on ice and superoxide anion release was assessed by scanning spectrophotometry (mean ± SEM, n = 3 in triplicate.

5.2.4 Adenosine Release

Having ruled out the possibility that the reversal of PAF-mediated priming was secondary to a decrease in neutrophil viability or activation potential, the degradation of cytochrome C, or the metabolism of PAF, the involvement of a paracrine "anti-inflammatory / anti-priming" mediator was next addressed. Since there have been many reports that adenosine can modulate various neutrophil responses, the potential generation of this mediator during the 2 hour incubation may have affected the priming induced by PAF and thereby contributed to the observed de-priming. Extracellular adenosine can be metabolized rapidly by the enzyme adenosine deaminase (ADA), and a concentration of 1 U/ml ADA has been shown to completely inhibit the effects of adenosine on neutrophils (McGarrity *et al.,* 1989; Bullough *et al.,* 1995). Therefore, by repeating the 2 hour incubation in the presence of 1 U/ml ADA, the effects of adenosine on the reversible priming induced by PAF could be evaluated.

5.2.4.1 Effects of Adenosine Deaminase on Superoxide Anion Release

Using either a 10 min or 120 min preincubation period, ADA was shown to have no effect on superoxide anion release from control or fMLP (100 nM)-stimulated neutrophils (Figure 5.4); however, ADA partially inhibited both the small superoxide response to 1 μM PAF alone and the PAF-primed fMLP response.

5.2.4.2 Effects of Adenosine Deaminase on Neutrophil Morphology

The effects of ADA on neutrophil morphology were also examined. Unlike its inhibition of superoxide anion responses, ADA did not alter the morphology of either control or PAF-treated (1 μM, 10 min) neutrophils. However, the shape change induced by fMLP (100 nM, 10 min) was inhibited by 39 ± 12% (Figure 5.5).

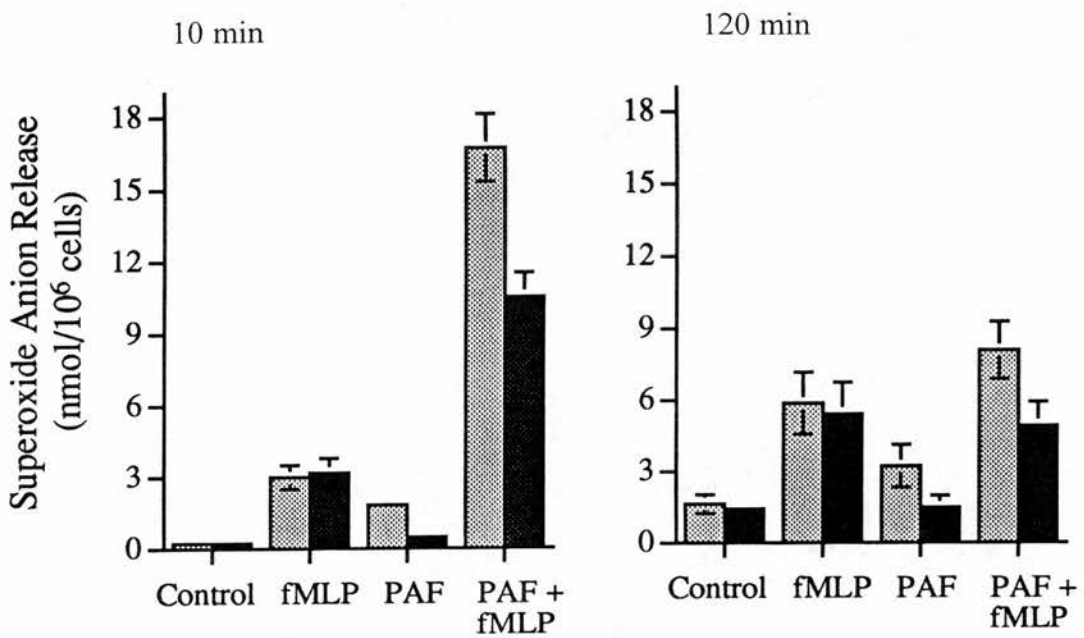


Figure 5.4
Effect of Adenosine Deaminase on Superoxide Anion Responses.
Neutrophils were incubated (± 1 U/ml adenosine deaminase) with PAF (1 μM) or buffer, for 10 min or 120 min. Following treatment with fMLP (100 nM, 10 min) or buffer, in the presence of 1 mg/ml cytochrome C, superoxide anion release was assessed by scanning spectrophotometry (mean ± S.E.M., n = 3 in duplicate).

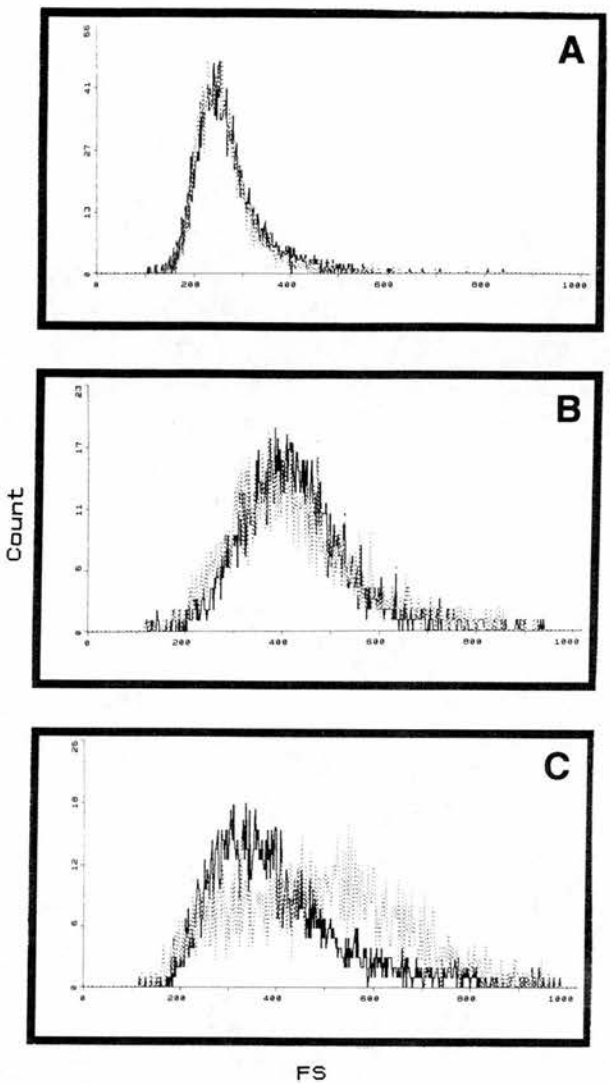


Figure 5.5
Effect of Adenosine Deaminase on Neutrophil Morphology.
Neutrophils were incubated with PAF (1 μM, 10 min, B), fMLP (100 nM, 10 min, C), or buffer control (A), in the presence (black outlines) or absence (light grey outlines) of 1 U/ml adenosine deaminase. Samples were analyzed for percent shape change by flow cytometry (x-axis: mean forward light scatter; FS; y-axis: relative cell number). Representative flow-cytometry (EPICS Profile II) histograms (n = 3 in triplicate).

In the absence of any direct toxic or non-specific inhibitory effects of ADA, these observations imply that adenosine is both present in the incubation medium and plays a role in enhancing the superoxide anion release of PAF-treated neutrophils, whilst playing no part in PAF-mediated polarization responses. This disagrees with a previous report in human neutrophils where adenosine completely inhibited the superoxide priming effect of 1 μM PAF (Stewart and Harris, 1993). In contrast, adenosine may facilitate fMLP-induced shape change, a finding which correlates with the increased chemotactic motility reported previously (Rose *et al.,* 1988; Garcia-Castro *et al.,* 1983). However, these findings do not support a role for adenosine release as a relevant mechanism underlying de-priming, since neutralizing its effects inhibited, rather than augmented, certain PAF-induced priming events.

5.2.5 Receptor-Dependency of PAF-Mediated Priming/De-priming Effects in Human Neutrophils

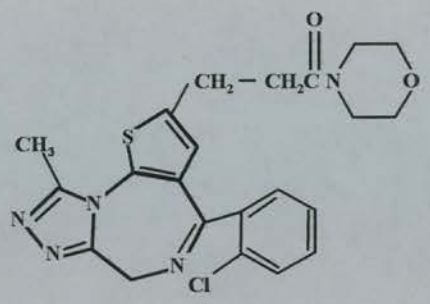
To ascertain whether the transient nature of PAF-induced priming reflected events occurring at a receptor (or sub-receptor) level, the effect of PAF receptor blockade on the priming and de-priming responses was investigated. From the large number of commercially-available PAF receptor antagonists, three compounds were selected that were specific and of high affinity, yet differed in their chemical structure (Figure 5.6), thereby eliminating any potential, structurally-dependent artefacts. Primarily, the thieno-triazolodiazepine WEB 2086 was chosen, as this compound has been one of the most widely-studied PAF receptor antagonists in a variety of model systems both *in vitro* and *in vivo* (Casals-Stenzel *et al.,* 1987). WEB 2086 has also been shown to antagonize PAF-induced responses in human neutrophils, including: aggregation (Casals-Stenzel *et al.,* 1987); chemotaxis (Fukuda *et al.,* 1989); degranulation (Dent *et al.,* 1989); and priming of the respiratory burst to fMLP (Pinckard and Prihoda, 1996; Gay, 1993). Secondly, the 1,4-dihydropyridine calcium channel blocker UK-74,505 was selected, since this agent has independent actions as a potent and long-lasting PAF receptor antagonist *in vivo* (Parry *et al.,*

1990). A direct structural analogue of PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine was also chosen.

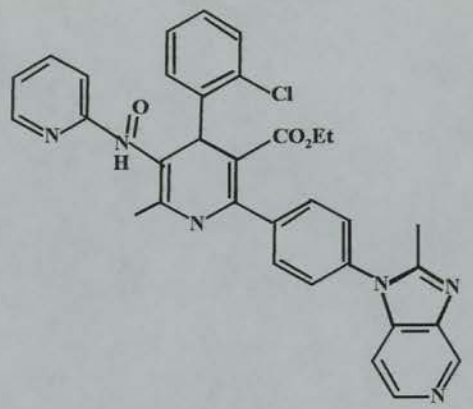
5.2.5.1 Concentration-Dependent Inhibition of Respiratory Burst Activity by PAF Receptor Antagonists

In order to assess whether the three selected compounds were effective PAF receptor antagonists in our system, and to determine appropriate drug concentrations for use in subsequent experiments, we examined their effects upon neutrophil viability and superoxide anion release in control and fMLP-treated neutrophils. At concentrations ≤10 μM, both WEB 2086 (Boehringer Ingelheim Ltd., Berks, UK) and UK-74,505 (a kind gift from Dr J. Parry, Pfizer, UK) had no effect on neutrophil viability or superoxide responses of control or 100 nM fMLP-stimulated neutrophils (Figure 5.7). These null effects of WEB 2086 are in agreement with a number of previous observations (Pinckard and Prihoda, 1996; Gay, 1993). However, the third antagonist tested, the PAF analogue 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine (Calbiochem, Nottingham, UK), displayed a number of direct and toxic effects upon neutrophils: at a concentration of 0.1 nM it increased basal and fMLP-stimulated superoxide responses (Figure 5.7); at concentrations ≥1 μM it reduced neutrophil viability by >50%; and at ≥10 μM it caused overt neutrophil lysis with DNA leakage. Thus, only WEB 2086 and UK-74,505 were studied further.

(1) WEB 2086



(2) UK-74,505



(3) 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine

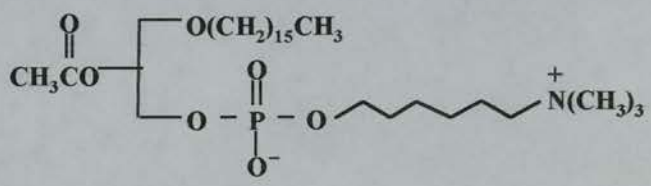


Figure 5.6
Molecular Structure of PAF Receptor Antagonists.

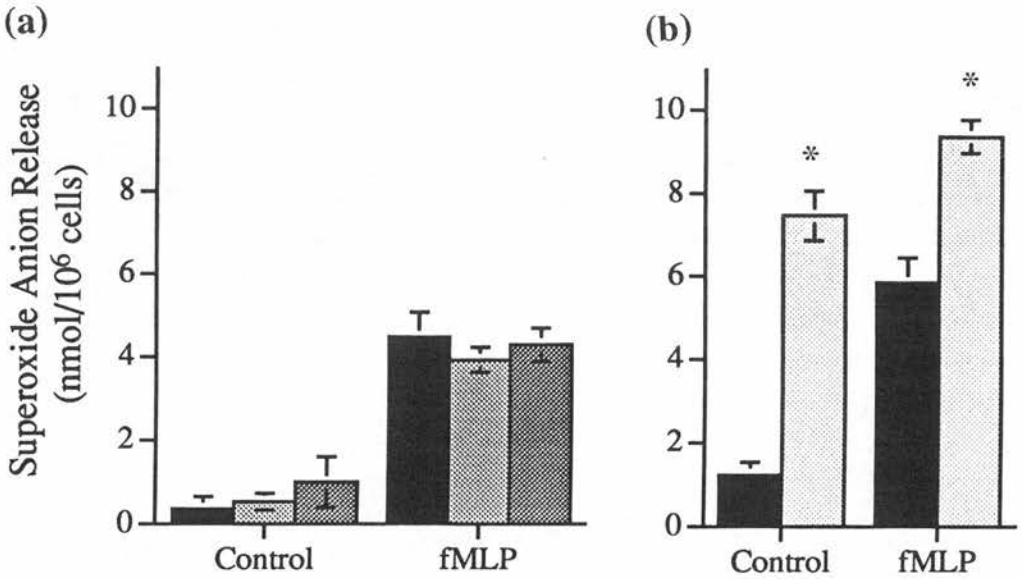


Figure 5.7
Effect of PAF Receptor Antagonists on Basal and fMLP-Stimulated Superoxide Anion Release in Human Neutrophils.
Neutrophils were pre-incubated for 30 min with: (a) WEB 2086 (10 μM, mid-grey bars), UK-74,505 (10 μM, dark-grey bars), buffer control (closed bars), or (b) 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine (0.1 nM, light bars). Following treatment with fMLP (100 nM, 10 min) or buffer control in the presence of 1 mg/ml cytochrome C, superoxide anion release was assessed by scanning spectrophotometry (mean ± SEM, n = 4 in triplicate). **P*<0.05, significantly different from values obtained in the absence of antagonist (ANOVA).

Preliminary studies were designed to establish the optimal concentrations of WEB 2086 and UK-74,505 for inhibition of PAF-induced responses of human neutrophils. When neutrophils were preincubated for 30 min with either antagonist, there was a concentration-dependent inhibition of the PAF-primed fMLP-stimulated superoxide response (Figure 5.8). The inhibition by UK-74,505 had an IC_{50} value of 68 ± 9 nM and was complete at 1 μM, whereas the inhibition by WEB 2086 was biphasic (reaching a plateau at 300 nM-1 μM) and incomplete at the highest concentration tested (55 ± 4% inhibition with 10 μM WEB 2086). This degree of antagonism of PAF-primed fMLP-stimulated superoxide anion release by WEB 2086 is similar to that previously reported (Gay, 1993).

As a further indication of the antagonistic capacity of WEB 2086 in human neutrophils, its ability to attenuate intracellular respiratory burst activity was also examined. Although WEB 2086 (1 μM, 30 min) caused a small increase (25%) in the DHR oxidation of control neutrophils, it had no significant effect on fMLP (100 nM)-stimulated respiratory burst activity (Figure 5.9). Furthermore, WEB 2086 inhibited the direct intracellular response to 1 μM PAF (yielding fluorescence values lower than WEB 2086-treated control neutrophils), and caused a small (28%) yet significant reduction in PAF-induced priming of the fMLP response.

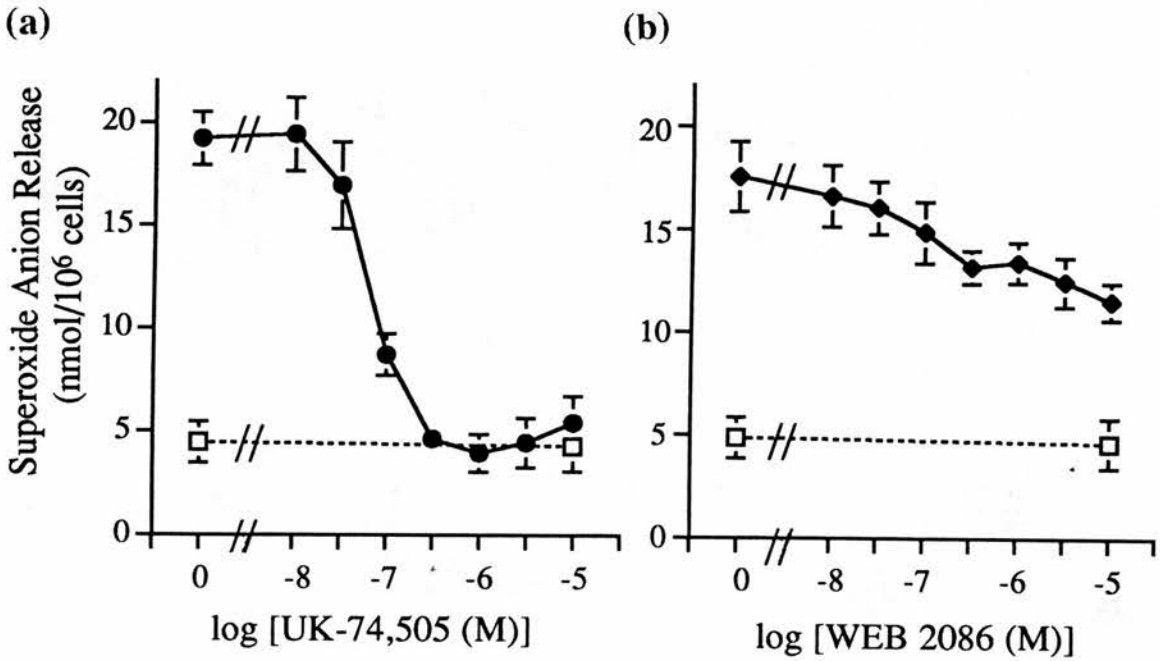


Figure 5.8
Concentration-Response of (a) UK-74,505 and (b) WEB-2086 on PAF-Induced Priming of fMLP-Stimulated Superoxide Anion Release in Human Neutrophils.
Neutrophils were pre-incubated for 30 min with: (a) UK-74,505 (10 nM-10 μM, closed circles), (b) WEB-2086 (10 nM-10 μM, closed diamonds), or buffer control. Following treatment with PAF (1 μM, 10 min) or buffer (open squares), neutrophils were incubated with fMLP (100 nM, 10 min) in the presence of 1 mg/ml cytochrome C, and superoxide anion release was assessed by scanning spectrophotometry (mean ± SEM, n = 4 in triplicate).

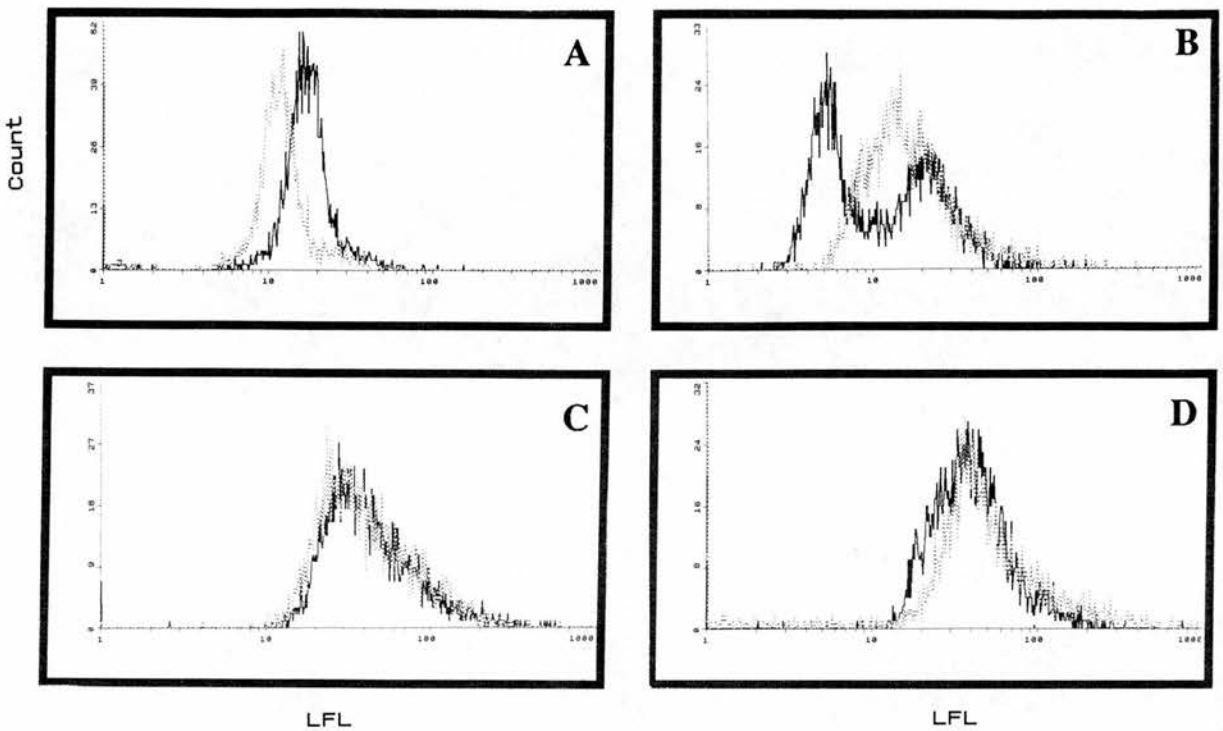


Figure 5.9
Effect of WEB 2086 on PAF-Induced Priming of fMLP-Stimulated Intracellular Respiratory Burst Activity.
Neutrophils were pre-incubated for 30 min with WEB-2086 (1 μM, black outlines) or buffer (light grey outlines), and then treated with PAF (1 μM, 10 min, B and D) or buffer (A and C) in the presence of 1 μM DHR. Following a further incubation with fMLP (100 nM, 10 min, C and D) or buffer (A and B), samples were analyzed by flow cytometry (x-axis: logarithmic scale green fluorescence, LFL; y-axis: relative cell number). Representative flow-cytometry (EPICS Profile II) histograms (n = 3 in duplicate).

5.2.5.2 Concentration-Dependent Inhibition of PAF-Induced Shape Change by PAF Receptor Antagonists

WEB 2086 was also used to inhibit neutrophil shape change induced by PAF. This enabled its inhibitory capacity to be quantified for an effector response that was due solely to PAF receptor activation and independent of other neutrophil stimulants. Following a 30 min pre-incubation with human neutrophils, WEB 2086 had no effect on the morphological appearance of control cells, but caused a concentration-dependent inhibition of PAF-induced shape change that was again biphasic and incomplete (73 ± 7%) up to 10 μM (Figure 5.10). Since [^{3}H]WEB 2086, at concentrations ≤200 nM, has been reported to bind specifically to a homogeneous population of high-affinity PAF receptors in human neutrophils (Dent *et al.*, 1989), the biphasic nature of the antagonism by WEB 2086 in our experiments may have represented binding, at concentrations >1 μM, to a lower-affinity subtype/conformational state of extracellular or intracellular PAF receptors (De Kimpe *et al.*, 1995; Stewart *et al.*, 1990).

5.2.5.3 The Reversal of the PAF-Primed Superoxide Response is Enhanced by PAF Receptor Antagonism

Having shown that pre-incubation of neutrophils with PAF receptor antagonists inhibited both PAF-induced superoxide priming and shape change, we next investigated whether the time-course for de-priming could be influenced by the subsequent blockade of PAF receptors. When either 1 μM WEB 2086 or 1 μM UK-74,505 was added 10 min after PAF (1 μM), there was a small, but significant, increase in the rate of decay of the PAF-primed superoxide anion response (Figure 5.11), with UK-74,505 having the greater effect. This implies that at least a proportion of PAF receptors remain in a functional state at the cell surface 10 min after the addition of PAF, and that the rate of de-priming may be influenced by the availability of functional PAF receptors.

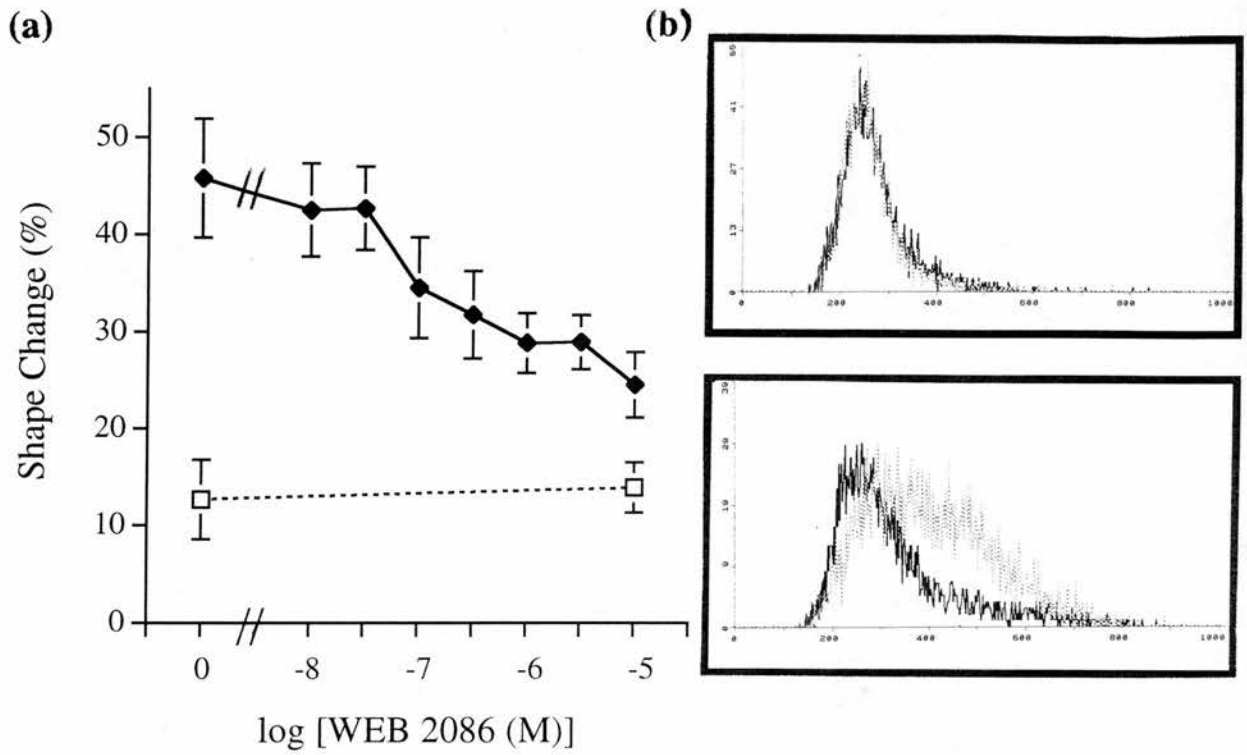


Figure 5.10
Concentration-Response of WEB-2086 on PAF-Induced Neutrophil Shape Change.
(a) Neutrophils were pre-incubated for 30 min with WEB-2086 (10 nM-10 μM, closed diamonds) or buffer control, and then treated with PAF (1 μM, 10 min) or buffer (open squares). Samples were analyzed for percent shape change by flow cytometry (mean ± SEM, n = 4 in triplicate). (b) Representative flow-cytometry (EPICS Profile II) histograms of neutrophils incubated with (A) 1 μM PAF or (B) 10 μM WEB 2086 then 1 μM PAF (x-axis: mean forward light scatter, FS; y-axis: relative cell number).

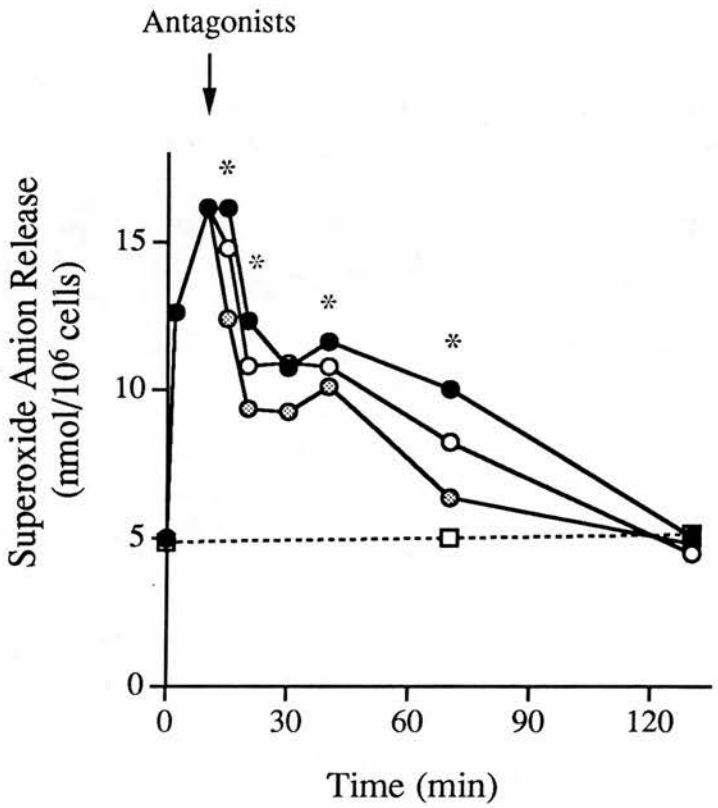


Figure 5.11
Effect of WEB 2086 and UK-74,505 on Time-Course for PAF-Induced Priming of fMLP-Stimulated Superoxide Anion Release in Human Neutrophils.
Neutrophils were incubated with PAF (1 μM, circles) or buffer (squares) for 0-120 min, with a further addition of WEB 2086 (1 μM, white circles), UK-74,505 (1 μM, grey circles) or buffer (black circles) 10 min after PAF. Following treatment with fMLP (100 nM, 10 min) in the presence of 1 mg/ml cytochrome C, superoxide anion release was assessed by scanning spectrophotometry (mean, n = 3 in triplicate. SEM values all <10% of mean and are omitted for reasons of clarity). *P<0.05, significantly different from values obtained in the absence of antagonist (ANOVA).

It should be noted that the biphasic pattern of de-priming was preserved in the presence of PAF receptor antagonists, again implicating a two-stage "recovery" mechanism (Chapter 4). WEB 2086 also inhibited the secondary increase (between 30-120 min) in PAF-mediated neutrophil shape change (data not shown).

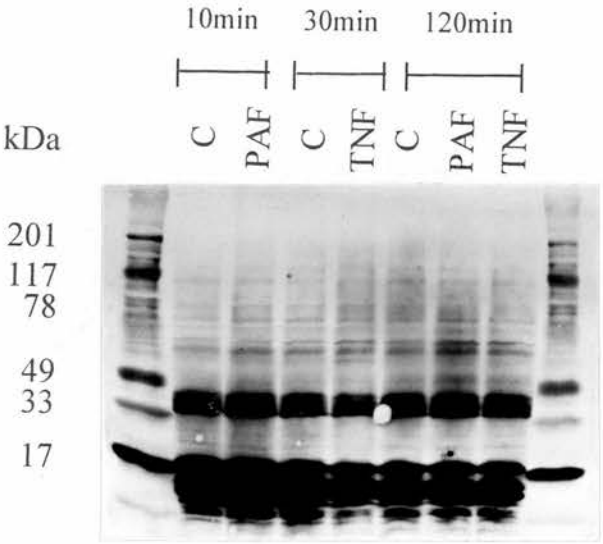
5.2.6 Tyrosine Phosphorylation of Intracellular Proteins

The stimulation of neutrophils by various priming agents has been reported to elicit the tyrosine phosphorylation of several intracellular proteins (Lloyds and Hallett, 1994; Kanbara *et al.,* 1993; Akimaru *et al.,* 1992; Lloyds *et al.,* 1995). Indeed, both PAF and C-PAF have been shown to phosphorylate the same group of proteins, in a concentration-, time-, and receptor-dependent manner (Gomez-Cambronero *et al.,* 1991). Some of these proteins are of similar molecular weight to those tyrosine phosphorylated when neutrophils are primed by hypotonic shock or TNFα (Edashige *et al.,* 1993). Furthermore, the time-course of tyrosine phosphorylation of a 115-kDa protein has been shown to correlate with the reversible, hypotonic priming of neutrophils (Edashige *et al.,* 1993): a similar protein (116-kDa) is affected within 2 min of PAF (100 nM) treatment, and remains phosphorylated for at least 10 min (Gomez-Cambronero *et al.,* 1991).

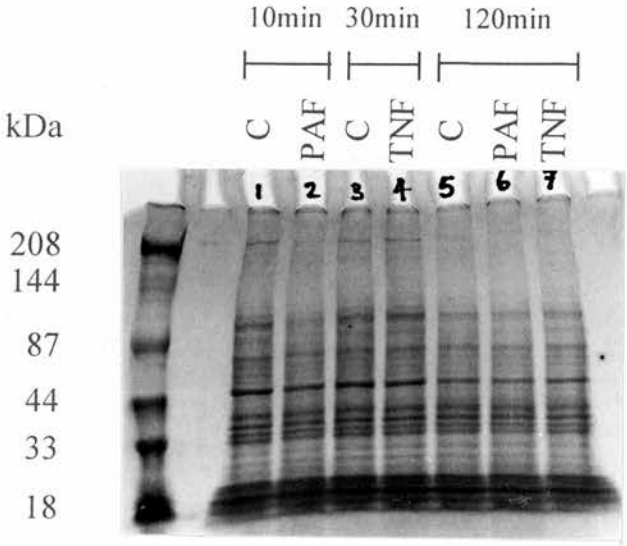
Therefore, by screening for proteins that were tyrosine phosphorylated in a transient manner in parallel with the functional (superoxide) priming effects of PAF, we ultimately aimed to identify specific proteins which were central to the priming process. Parallel incubations were performed with TNFα, a neutrophil priming agent that does not signal through the G-protein-linked receptor family utilized by classical chemoattractants. PAF (1 μM, 10 min) and TNFα (100U/ml, 30 min) both primed the fMLP-superoxide anion response of neutrophils: an effect that was more transient with PAF than TNFα (Figure 5.12). However, despite detection of a number of consistent, tyrosine-phosphorylated protein bands (using a monoclonal anti-phosphotyrosine antibody), there was no detectable difference between the tyrosine

phosphorylation levels of control, primed or de-primed neutrophils. This finding was reproduced in eight out of nine experiments, all controlled for protein concentration (assessed by the Coomassie Brilliant Blue staining of parallel gels). In the remaining experiment, a protein of approximately 120-kDa was tyrosine phosphorylated in primed (PAF 10 min, TNFα 30 min, and less so with TNFα 120 min), but not in control or de-primed (PAF 120 min), neutrophils. Although this may have represented the 115-116-kDa protein that has previously been associated with neutrophil priming (Gomez-Cambronero *et al.,* 1991; Edashige *et al.,* 1993), it was not a consistent association in our hands. Therefore, in an attempt to identify whether our protein extraction or detection methodolgy was at fault, identical experiments were repeated by Dr. V. Cherepanov and Professor G. Downey (Dept. of Medicine, University of Toronto, Canada) using an alternative immunoblotting protocol: again, optimal priming incubations with both PAF (10 min) and TNFα (30 min) failed to increase phospho-tyrosine levels in human neutrophils.

(a)



(b)



(c)

	10 min	30 min	120 min
Control	4.89 ± 0.57	4.78 ± 0.46	5.14 ± 0.73
PAF	16.06 ±1.35	n/a	8.20 ± 1.21
TNFα	n/a	23.44 ± 3.19	21.90 ± 2.45

Figure 5.12
Effect of PAF and TNFα on Neutrophil Protein Tyrosine Phosphorylation.

(a) Representative Immunoblot of Phosphorylated Tyrosine Residues. Neutrophils were incubated. with PAF (1 μM), TNFα (100 U/ml) or buffer for 10-120 min. Reactions were stopped at the appropriate times with 20% TCA, and PAGE was performed using 4-20% Tris-glycine gels. Following transfer to nitrocellulose membranes, proteins were immunoblotted with monoclonal anti-phosphotyrosine antibody (4G10), and visualized by ECL. (b) Parallel gel stained with Coomassie Brilliant Blue. (c) Parallel superoxide anion responses. Neutrophils were primed with PAF (1 μM) or TNFα (100 /ml) for 10-120 min, prior to treatment with fMLP (100 nM 10 min) in the presence of 1 mg/ml cytochrome C. Superoxide anion release was assessed spectrophotometrically (mean ± SEM for triplicate determination of representative experiment of 8).

5.3 Discussion

While the basis for the decline in PAF-mediated priming is uncertain, the maintenance of PMA responsiveness and re-priming potential indicate that it does not reflect a diminished viablity or superoxide anion-generating capacity of human neutrophils. However, neutrophils can synthesize a variety of inflammatory mediators, some of which may act in a paracrine fashion to modulate neutrophil responses: it was considered that such an agent with inhibitory properties might contribute to the reversal of PAF-mediated neutrophil priming. Since adenosine is a product of normal cellular activity that has been shown to inhibit both respiratory burst and adhesion responses of human neutrophils (Cronstein *et al.,* 1987; Stewart and Harris, 1993; Walker *et al.,* 1990; Ward *et al.,* 1988; Cronstein *et al.,* 1983; Cronstein *et al.,* 1992; Asako *et al.,* 1993), it seemed an ideal candidate for this role. However, the inclusion of adenosine deaminase (which degrades extracellular adenosine) during the 2 hour neutrophil incubation with PAF, failed to maintain PAF-mediated priming of fMLP-stimulated superoxide anion release. These data contrast with a previous report where adenosine inhibited the superoxide priming effect of 1 μM PAF (Stewart and Harris, 1993). It should be noted, however, that the neutrophils used in this latter study were primed at the beginning of the assay, and that this effect was also inhibited by adenosine. Basal neutrophil priming is a confounding factor in many neutrophil activation studies and its importance in the *ex vivo* manipulation of neutrophils is often underestimated. Nevertheless, the neutrophil responses elicited by PAF may be modulated by other inflammatory mediators, such as LTB_4 or PGE_2, but this possibility has not been pursued.

The two, structurally-unrelated PAF receptor antagonists, UK-74,505 and WEB 2086, were shown to suppress the priming effects of PAF. However, the biphasic, partial inhibitory effects of WEB 2086 (≤10 μM) implied that, unlike UK-74,505, this compound was having complex effects, possibly via interaction with a non-homogeneous population of PAF receptors. Although [^{3}H]WEB 2086 has been

reported to bind (K_D 18.9 nM) to a homogeneous population of non-interacting binding sites in human neutrophils when used at concentrations ≤200 nM (Dent *et al.,* 1989), it has since been proposed that the hydrophilic nature of WEB 2086 might allow it to traverse neutrophil membranes (at higher concentrations), and thereby inhibit both the extracellular and the putative intracellular PAF receptor (Koike *et al.,* 1994; De Kimpe *et al.,* 1995; Stewart *et al.,* 1990). Since various priming agents, including PAF (Doebber and Wu, 1987) and C-PAF (Tessner *et al.,* 1989), have been shown to promote PAF synthesis in human neutrophils (De Nichilo *et al.,* 1991; Wirthmueller *et al.,* 1989; Stewart and Harris, 1991; Stewart *et al.,* 1991; Worthen *et al.,* 1988), a role for intracellularly-retained PAF in neutrophil priming cannot be excluded (Pabst, 1994).

Previous groups have demonstrated that exogenous PAF mediates its effects through high-affinity receptors on the neutrophil surface; it is therefore possible that these receptors play a central role in the reversal of PAF-induced responses. Indeed, the functional uncoupling and subsequent down-regulation of cell-surface PAF receptors has been proposed to underlie the homologous desensitization that occurs when neutrophils are repeatedly exposed to PAF (O'Flaherty *et al.,* 1992). PAF receptor desensitization is rapid, occurring maximally within 15 s of PAF addition (0.1-10 nM) (O'Flaherty *et al.,* 1992). However, the ability of UK-74,505 and WEB 2086 to increase the rate of de-priming following PAF treatment suggests that a population of PAF receptors remains functionally active for at least 10 min. PAF receptor desensitization is transient, with neutrophils beginning to recover their sensitivity to PAF and regain high-affinity PAF receptors within approximately 10 min of PAF exposure, a process which is completed by 60 min (O'Flaherty *et al.,* 1992). This explains why a second PAF challenge could re-prime the fMLP-superoxide response after 2 hours, with no significant reduction in its priming potential (Chapter 4). Furthermore, since TNFα could also re-prime PAF-recovered neutrophils, any heterologous desensitization to TNFα had reversed within 2 hours. Heterologous desensitization to fMLP has been shown not to occur (Gay, 1993; O'Flaherty *et al.,* 1992) and thus can be excluded as a potential mechanism of de-priming.

Although binding studies have provided clear evidence that human neutrophils express specific, high-affinity PAF receptors, they have also demonstrated high levels of non-specific PAF binding to neutrophil plasma membranes (Bussolino *et al.,* 1984; O'Flaherty *et al.,* 1986). More recently, it has been shown that the internalization of PAF occurs via a receptor-independent, non-endocytic process, which may involve the "flipping" of intact PAF across the perturbed plasma membrane of stimulated neutrophils (Bratton *et al.,* 1992). Furthermore, this uptake process appears to be the rate-limiting step in PAF metabolism (Bratton *et al.,* 1992; Tokumura *et al.,* 1990). Therefore, it was proposed that PAF may initially associate with the external leaflet of the neutrophil plasma membrane, before either binding to its high-affinity receptor or being internalized for subsequent metabolism (O'Flaherty *et al.,* 1992). The internalization of PAF may sequester ligand from specific PAF receptors and indirectly promote its intramembranous metabolism and subsequent removal to granular membranes (O'Flaherty *et al.,* 1986). Thus, internalization may represent a mechanism aside from homologous receptor desensitization that limits the stimulation of neutrophils by PAF. Although PAF internalization begins within approximately 2 min of PAF accumulating on the neutrophil surface (O'Flaherty *et al.,* 1992), it is a more gradual process than receptor desensitization, and requires at least 20 min with PAF (200 pM-75 nM) to near completion (Bratton *et al.,* 1992; O'Flaherty *et al.,* 1986). However, the capacity of neutrophils to internalize PAF increases with increasing concentrations of extracellular PAF (O'Flaherty *et al.,* 1986). Therefore, the continual clearance of PAF from the immediate vicinity of neutrophils may provide a mechanism to limit re-stimulation of neutrophils through newly-expressed PAF receptors (O'Flaherty *et al.,* 1992).

This putative desensitization-internalization-resensitization sequence may underlie the reversal of PAF-mediated priming. For example, priming may be initiated by the stimulation of PAF receptors on the neutrophil surface, which uncouple rapidly from their signal transduction pathways once the signal has been transmitted to the cell interior: whilst neutrophil priming is becoming established and apparent, PAF

receptors become quantitatively down-regulated (O'Flaherty *et al.,* 1992). After approximately 10 min, neutrophils begin to recover their sensitivity to PAF as high-affinity PAF receptors are either being re-expressed or uncovered. However, since PAF may have been cleared by internalization at this stage, the amount of ligand available for receptor occupancy is much reduced. If the intracellular signalling pathways that mediate PAF-induced priming are subject to feedback mechanisms or are short-lived and spontaneously abort, then priming will reverse unless a new stimulus is applied to the cell. Therefore, as the extent of priming induced by PAF appears to be linked directly to receptor occupancy (as implicated by its concentration-dependence (Chapter 4)), priming will decline (intracellular signalling permitted) as the extracellular concentration of PAF diminishes. This would explain the increased rate of de-priming: (i) upon PAF receptor blockade with UK-74,505 and WEB 2086; and (ii) with a lower concentration of 10 nM PAF (Vercellotti *et al.,* 1988).

Despite such arguments, the identical, transient time-courses of superoxide priming by PAF and C-PAF (a biologically active, yet non-metabolizable, analogue of PAF), imply that the metabolism of PAF does not form the basis of the de-priming process. Indeed, since C-PAF can also be internalized by neutrophils (Bratton *et al.,* 1992) and its biological potency correlates precisely with its binding affinity for the PAF receptor (O'Flaherty *et al.,* 1987), it is possible that the internalization, rather than the metabolism, of PAF is the process which limits the duration of PAF-mediated priming. However, the biphasic nature of superoxide de-priming following PAF treatment suggests that two different mechanisms may be involved: since the second, slower phase of decay occurred concurrently with the delayed, secondary increase in neutrophil shape change (an event which was also inhibited by WEB 2086), the same PAF receptor-dependent mechanisms may underlie both events.

The intracellular signalling mechanisms involved in neutrophil priming remain uncertain, making it impossible to predict the mechanisms which contribute to the reversal of this effect. Nevertheless, the time- and concentration-dependence of

certain PAF-induced intracellular events have been shown to correlate with functional priming responses. For example, PAF-induced priming of fMLP-stimulated superoxide anion release concurs with: (i) the translocation of $G_{i\alpha 2}$ to the neutrophil plasma membrane (Alison Condliffe, personal communication); and (ii) the tyrosine phosphorylation of several intracellular proteins (Nick *et al.,* 1997; Gomez-Cambronero *et al.,* 1991). However, we were unable to confirm this latter observation and hence could not establish a role for reversible protein tyrosine phosphorylation in the transient neutrophil priming effects of PAF.

Other intracellular events, such as PKC activation (Gay, 1993; Gay and Stitt, 1988; O'Flaherty and Nishihira, 1987) and $[Ca^{2+}]_i$ elevation (Ingraham *et al.,* 1982), have also been associated with PAF-mediated neutrophil priming, although Ca^{2+}-independent pathways of priming may also exist (Gay, 1993; Walker *et al.,* 1991; Koenderman *et al.,* 1989). The activation of phospholipase A_2 and D (Kanaho *et al.,* 1991; Nakashima *et al.,* 1989), phosphoinositide turnover (Naccache, 1985) and intracellular alkalinization (Naccache *et al.,* 1986), also occur in PAF-treated neutrophils. Thus, the subsequent reversibility/inhibition of any of the above events may contribute to the transient nature of the priming effects of PAF. Furthermore, the regulation of divergent intracellular signalling pathways may dictate the rate of onset and subsequent duration of: (i) different priming-associated responses (e.g. NADPH oxidase activity and cell polarization); and (ii) the priming effects of individual agents (e.g. LPS, G-CSF and PAF).

In conclusion, the mechanisms underlying the reversible priming effects of PAF remain uncertain. However, a diminished viablity or superoxide anion-generating capacity of human neutrophils is not involved. In addition, de-priming is not solely secondary to the metabolism of PAF, the paracrine effects of adenosine, or homologous receptor desensitization (since PAF receptor antagonists increased the rate of decay). The transient priming effects of PAF are most likely to reflect reversal of intracellular events: the tyrosine phosphorylation of specific proteins and the translocation of G-protein subunits to the plasma membrane warrant further investigation.

6. CHAPTER 6: THE PRIMING OF HUMAN NEUTROPHILS BY TUMOUR NECROSIS FACTOR α

6.1 Introduction

The studies with PAF (Chapters 4 and 5) have provided clear evidence of receptor-mediated priming that spontaneously and completely reverses. The primary aim of the work in this Chapter was to establish whether the reported, more sustained, priming effects of other pro-inflammatory mediators could be manipulated and reversed.

PAF, $InsP_6$ and hypotonic shock all prime neutrophils rapidly, which contrasts with LPS (Guthrie *et al.,* 1984; Condliffe *et al.,* 1996), GM-CSF (Weisbart *et al.,* 1985) and IFN-γ (Roberts *et al.,* 1993) which all require at least one hour to elicit their maximal priming effects. Since these latter agents are also amongst those reported to have the most prolonged priming effects in neutrophils (Roberts *et al.,* 1993; Carey *et al.,* 1994; Ichinose *et al.,* 1990), this suggests that the rate of onset of neutrophil priming may determine its subsequent reversibility. However, neutrophils have a relatively short life-span *in vitro* due to their high rate of constitutive apoptosis, and together with the long pre-incubation times required for LPS, GM-CSF and IFN-γ to induce their priming effects, this limits the subsequent manipulation of these responses. It is also very difficult (as observed in our shape change experiments, Chapter 4) to prevent some degree of spontaneous priming in neutrophils incubated for >60 min *ex vivo*. Thus, a priming agent with a pre-incubation period longer than that of PAF, but ideally less than one hour was required.

TNFα is an established pro-inflammatory agent (Berkow *et al.,* 1987; Larrick *et al.,* 1987) that has been reported to elicit its optimal, receptor-mediated priming effects within 30 min of exposure to suspension neutrophils (Condliffe *et al.,* 1996; Elbim *et*

al., 1993; Roberts *et al.,* 1993). Furthermore, it has been shown that the *in vitro* priming effects of TNFα are long-lived. For example, priming of fMLP-stimulated superoxide anion release has been reported to persist for at least 4 hours following TNFα treatment (Ferrante *et al.,* 1988), potentiation of the opsonised zymosan-induced respiratory burst to be maintained for at least 90 min (Ozaki *et al.,* 1988), and enhancement of fMLP-induced degranulation to be preserved for at least 1 hour (O'Flaherty *et al.,* 1991). In addition, neutrophil shape change induced by TNFα has been reported to remain unaltered for at least 50 min (Shimizu *et al.,* 1993). It should be noted that these times all represent the end of the respective study periods and no attempt was made to follow these effects over a longer time-period. Therefore, TNFα was selected as the receptor-mediated agent of choice whose priming effects were sufficiently rapid, robust and sustained, to allow examination of the potential for primed neutrophils to be "artificially" de-primed.

It was postulated in Chapter 5 that the duration of neutrophil priming might be linked to the duration of cell-surface receptor occupancy, and that the spontaneous reversal or negative-feedback regulation of intracellular signalling pathways might allow termination of priming events, unless continual stimulatory signals were received by the cell. This proposal was based on several observations, namely that: (i) the specific blockade of PAF receptors (with two, structurally-different PAF receptor antagonists) could increase the rate of reversibility of the superoxide priming effect of PAF; (ii) the priming induced by a lower concentration of 10 nM PAF (Vercellotti *et al.,* 1988) appeared to be more transient than that observed with 1 μM PAF in our studies; and (iii) the shape change induced by fMLP and C5a persisted until the stimulus was removed by washing (Smith *et al.,* 1979). Therefore, our initial experiments were designed to adopt such strategies in an attempt to manipulate the duration of TNFα-induced neutrophil priming.

TNFα signals through two distinct receptor subtypes, of molecular masses 55 kDa (TNF-R55) and 75 kDa (TNF-R75) (Brockhaus *et al.,* 1990). However, since certain

TNF-R-directed antibodies can fully mimic the activities of TNFα (especially following their crosslinking) (Bigda *et al.,* 1994; Espevik *et al.,* 1990), it has been suggested that the sole function of the native, trimeric TNFα ligand is to elicit receptor oligomerization. Despite this, a complete lack of homology between the intracellular domains of the two TNF-Rs implicates a divergence in both their signalling mechanisms and their biological function (Tartaglia and Goeddel, 1992; Dembic *et al.,* 1990). Specific TNF-R antagonistic antibodies have been used to examine the independent effects of the two receptors. TNF-R55 appears to be the predominant mediator of the majority of the biological effects of TNFα (Tartaglia and Goeddel, 1992; Barbara *et al.,* 1994; Loetscher *et al.,* 1993; Espevik *et al.,* 1990), including neutrophil priming (Barbara *et al.,* 1994; Abe *et al.,* 1995). In systems where inhibition of TNF-R75 does attenuate the functional effects of TNFα, a ligand passing model has been proposed (Tartaglia and Goeddel, 1992) in which TNF-R75 (having the higher affinity and dissociation rate for TNFα) preferentially binds TNFα and then passes it to neighbouring TNF-R55 to mediate its biological effects. However, the exact functional relationship between the two receptors is still unclear.

Therefore, the central aim of the work in this Chapter was to establish and characterize a TNFα-induced model of sustained neutrophil priming, in order to investigate whether such priming was also potentially reversible. In addition, the availability of subtype-specific TNF-R blocking antibodies allowed examination of the individual contributions made by TNF-R55 and TNF-R75 to the maintenance of the TNFα-induced primed state.

6.2 Results

6.2.1 The Reversibility of TNFα-Induced Priming of Human Neutrophils

In Chapter 4, it was established that TNFα could elicit a number of priming-associated responses in human neutrophils, including shape change, adhesion molecule upregulation, and priming of both superoxide anion release and intracellular respiratory burst activity to fMLP. Based upon consistent reports of its optimal priming effects in human neutrophils, a 30 min preincubation with TNFα was used for these preliminary studies (Condliffe *et al.*, 1996; Elbim *et al.*, 1993; Roberts *et al.*, 1993). However, since TNFα also has the capacity to directly activate neutrophils that are *adherent* (Nathan, 1987), the inadequate suspension of neutrophils may explain the marked variability that exists in the literature regarding the concentration-dependence and priming potential of TNFα *in vitro*. Therefore, in order to minimize adherence, neutrophils were incubated at low cell density in round-bottomed, polypropylene Eppendorf tubes and shaken continuously, yet gently (110 cycles/min), for the duration of the assay. Both the macroscopic and light-microscopic evaluation of these neutrophils revealed that they remained in suspension (i.e. did not become adherent or form cell aggregates) for at least 2 hours.

6.2.1.1 Time-Course for the Induction of Neutrophil Shape Change by TNFα

The induction of neutrophil shape change by PAF was shown to be the most rapid and transient of the three indices of priming (Chapter 4). This suggested that the polarization response might provide the earliest indication of whether the priming elicited by a particular agent would/could reverse. Therefore, as a verification of the prolonged actions of TNFα in human neutrophils, the time-course for the induction of shape change was examined. When neutrophils were incubated with 200 U/ml TNFα they underwent a gradual change in shape that reached a plateau (70 ± 5%) within 30 min and was maintained for at least 2 hours (Figure 6.1). This

demonstration of a persistent polarization response to TNFα confirms a previous observation (Shimizu *et al.*, 1993).

6.2.1.2 Time-Course for the Functional Upregulation of CD11b/CD18 by TNFα

As a second assessment of the stability of the priming effect of TNFα, the time-course for the functional upregulation of CD11b/CD18 was determined. The number of neutrophils with surface-bound ACLB was maximal (50 ± 6%) within 30-60 min exposure to 200 U/ml TNFα (Figure 6.2). Although this absolute effect (i.e. the percentage of neutrophils with attached beads) was maintained thereafter, there was a reproducible shift in the number of ACLB bound per neutrophil, so that after 2 hours the majority of neutrophils had 1-2 beads on their surface, in contrast to the 30 min and 60 min time-points where a higher number of beads bound per cell was common. Thus, whilst the number of neutrophils with functionally upregulated CD11b/CD18 was maintained, the overall "effectiveness" of CD11b/CD18-ACLB interactions on these cells (which may reflect CD11b/CD18 affinity, avidity or expression) showed signs of decaying between 60 min and 120 min post TNFα exposure. This implies that the functional upregulation of CD11b/CD18 by TNFα, at the single cell level, may be more transient than the accompanying neutrophil shape change response.

6.2.1.3 The Priming of fMLP-Induced Superoxide Anion Release by TNFα

As the *gold standard* indicator of neutrophil priming, the duration of the enhancement of fMLP-stimulated superoxide anion release was next investigated. Initially, it was important to establish the optimal priming concentration of TNFα to be used in these studies. A 30 min incubation with TNFα (0.1-1000 U/ml) elicited minimal direct superoxide anion release from human neutrophils (verifying the lack of cell adhesion), but caused a concentration-dependent enhancement (EC_{50} 21 ± 3 U/ml) of the subsequent superoxide response to fMLP (100 nM, 10 min) that was maximal with ≥100 U/ml TNFα (Figure 6.3). Therefore, a concentration of 100 U/ml TNFα was chosen for all further priming studies.

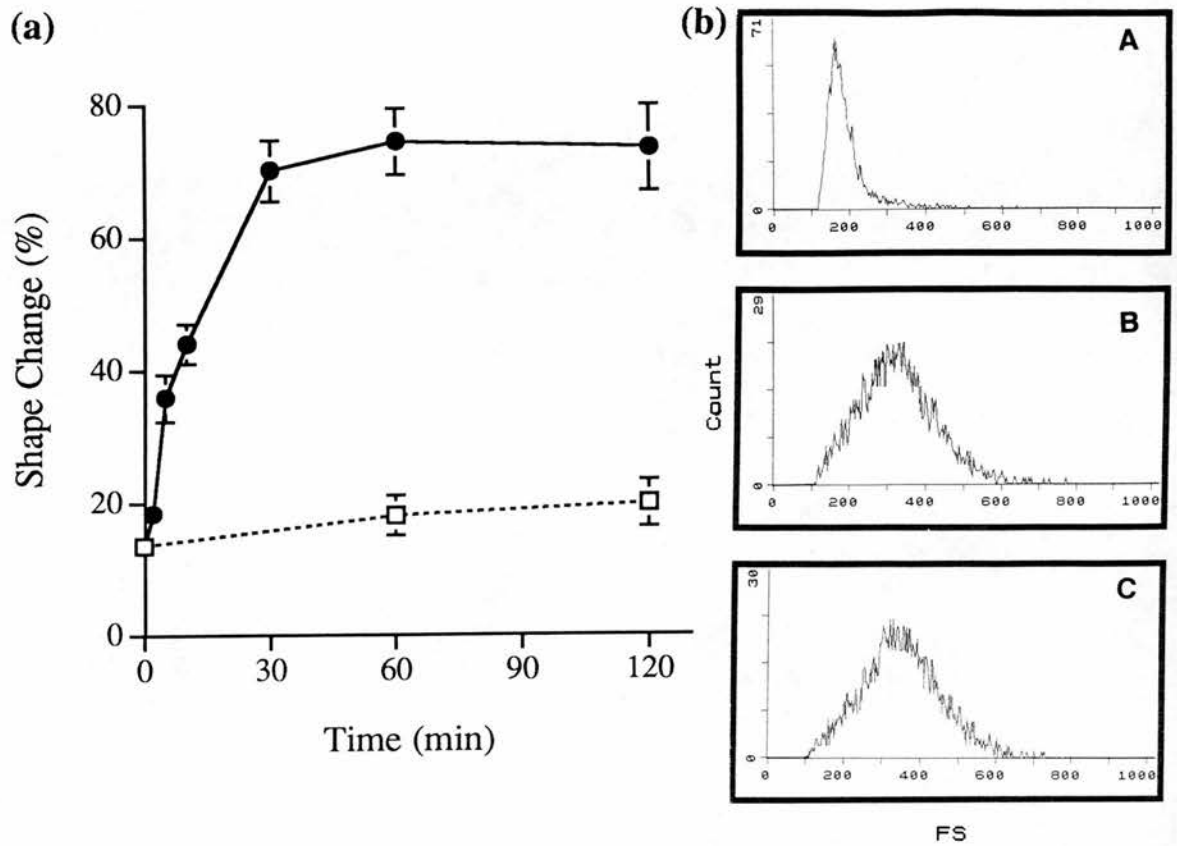


Figure 6.1

Time-Course for TNFα-Induced Shape Change in Human Neutrophils.

(a) Time-course for TNFα-induced shape change. Neutrophils were incubated with TNFα (200 U/ml, closed circles) or buffer (open squares) for 0-120 min. Samples were analyzed for percent shape change by flow cytometry (mean ± SEM, n = 4 in duplicate. Where not shown, SEM values fall within symbols). (b) Representative flow-cytometry (EPICS Profile II) histograms of control neutrophils (A) and neutrophils incubated with TNFα for 30 min (B) or 120 min (C) (x-axis: mean forward light scatter, FS; y-axis: relative cell number).

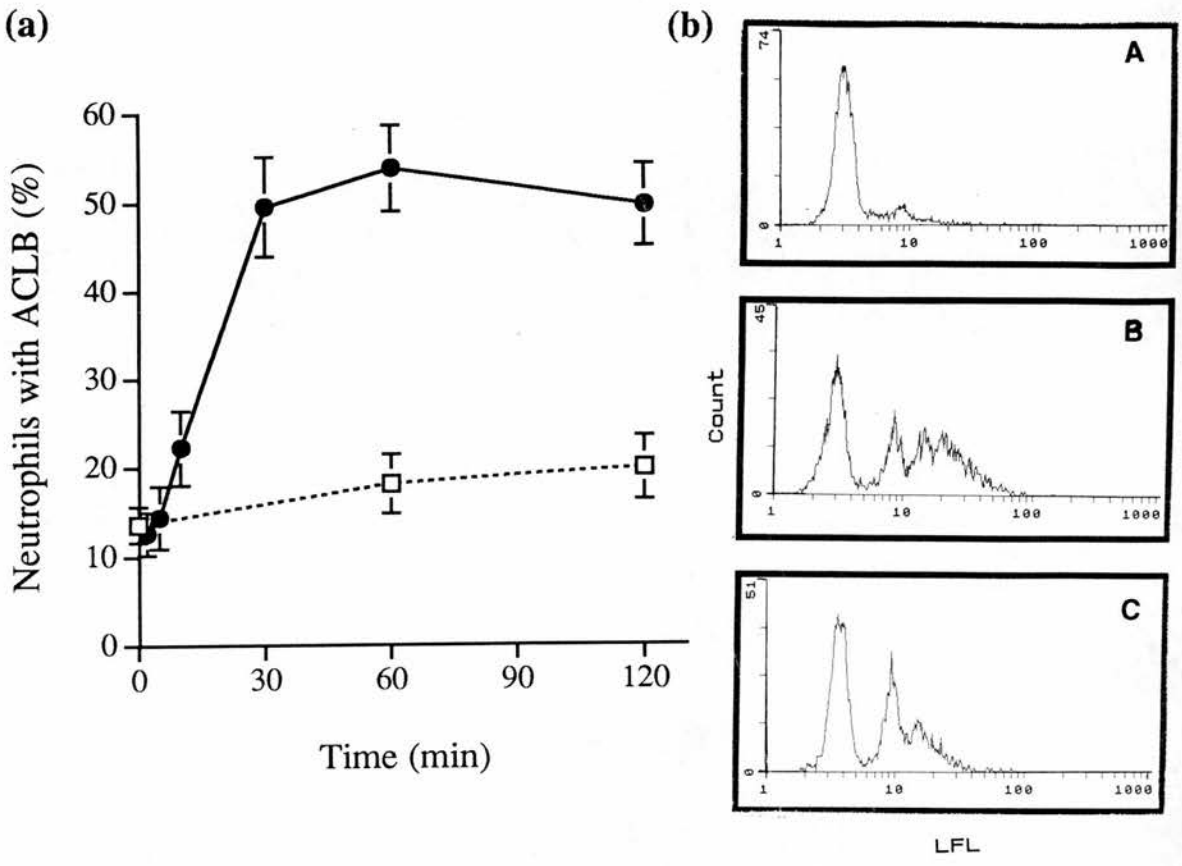


Figure 6.2

Time-Course for TNFα-Induced Binding of ACLB in Human Neutrophils.

(a) Time-course for TNFα-induced binding of ACLB. Neutrophils were incubated with TNFα (200 U/ml, closed circles) or buffer (open squares) for 0-120 min. ACLB (0.75% v/v) were added 15 min before the termination of the reaction with 0.5% glutaraldehyde, except for time-points <15 min where beads were added before the agonist. Samples were analyzed for attached ACLB by flow cytometry (mean ± SEM, n = 4 in duplicate. Where not shown, SEM values fall within symbols). (b) Representative flow-cytometry (EPICS Profile II) histograms of control neutrophils (A) and neutrophils incubated with TNFα for 60 min (B) or 120 min (C) (x-axis: logarithmic scale green fluorescence, LFL; y-axis: relative cell number).

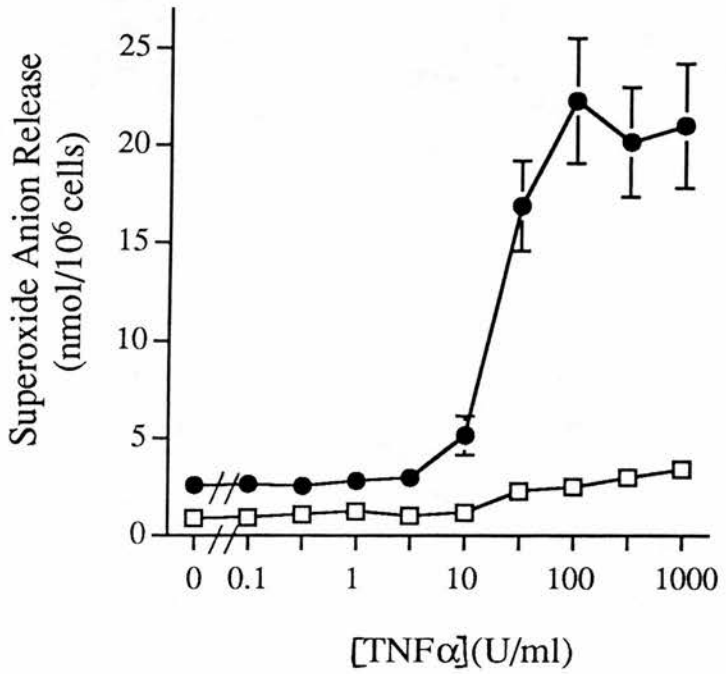


Figure 6.3

Concentration-Response for TNFα-Induced Priming of fMLP-Stimulated Superoxide Anion Release in Human Neutrophils.

Neutrophils were incubated with TNFα (0.1-1000U/ml, 30 min) or buffer, and then treated with fMLP (100 nM, 10 min, closed circles) or buffer control (open squares) in the presence of 1 mg/ml cytochrome C. Superoxide anion release was assessed by scanning spectrophotometry (mean ± SEM, n = 3 in duplicate).

6.2.1.4 Time-course for the Priming of Superoxide Anion Release by TNFα

In previous experiments (e.g. Figure 5.13), the effect of TNFα on fMLP-stimulated superoxide anion release was examined only at very selected time-points. Hence, a far more detailed time-course was required to determine the precise kinetics and persistence of this priming effect.

In agreement with previous studies (O'Flaherty *et al.,* 1991; Condliffe *et al.,* 1996), the priming of human neutrophils for fMLP-stimulated (100 nM, 10 min) superoxide anion release reached a plateau following 30 min exposure to 100 U/ml TNFα (Figure 6.4a). Although this response was maintained for 2 hours in certain individuals, the maintenance of the priming effect was highly variable with decreases, no change, or even further increases (30-120 min) in the level of enhancement. This variability did not reflect inter-assay differences in either the basal level of neutrophil priming or the absolute degree of priming elicited after a 30 min treatment with TNFα (Figure 6.4b). Thus, the duration of the superoxide-priming effect of TNFα appeared to be donor-dependent. It should also be noted that where TNFα-induced priming did reverse spontaneously, this differed from the pattern observed following PAF exposure: (i) irrespective of the rate of decay, the pattern was exponential and never biphasic; and (ii) a residual priming effect was always retained, even after an extended TNFα preincubation period of 150 min (data not shown).

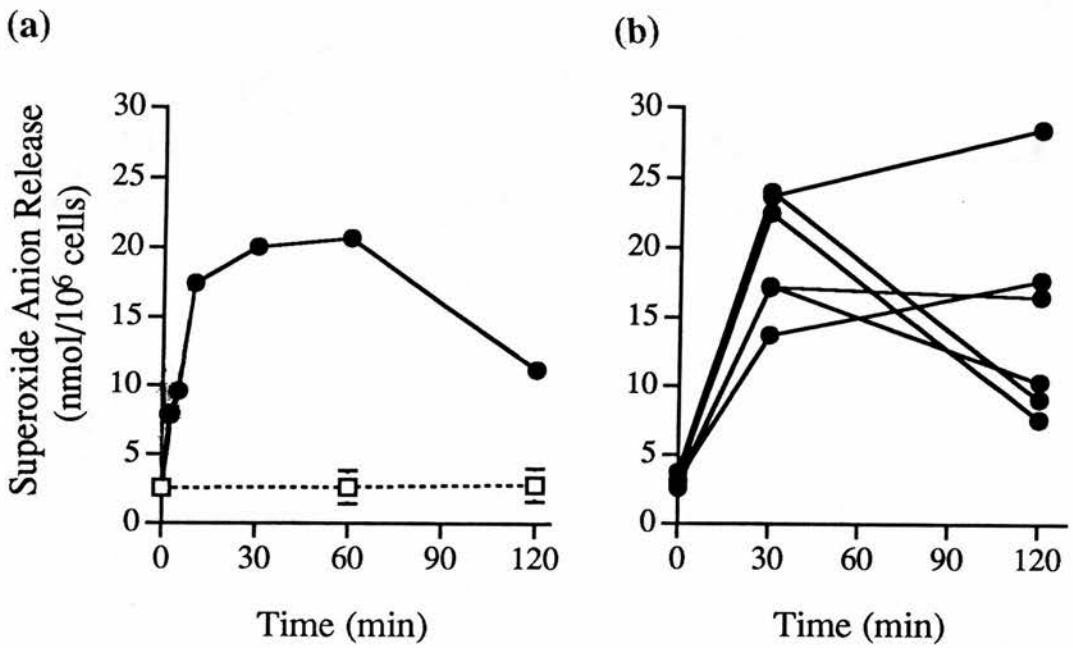


Figure 6.4
Time-Course for TNFα-Induced Priming of fMLP-Stimulated Superoxide Anion Release in Human Neutrophils.
(a) Representative time-course for TNFα-induced superoxide priming. Neutrophils were incubated with TNFα (100 U/ml, closed circles) or buffer (open squares) for 0-120 min. Following treatment with fMLP (100 nM, 10 min) in the presence of 1 mg/ml cytochrome C, superoxide anion release was assessed by scanning spectrophotometry (mean of triplicate determination from single experiment). (b) Variability in the degree and duration of TNFα-induced priming of fMLP-stimulated superoxide anion release (mean of triplicate determinations from 6 experiments).

6.2.1.5 Summary of the Time-Courses for TNFα-Induced Priming Effects in Human Neutrophils

(i) TNFα elicits its maximal effects on neutrophil morphology after 30 min, and this shape change effect is maintained for at least 2 hours.

(ii) The functional upregulation of CD11b/CD18 is maximal within 30-60 min exposure to TNFα. Thereafter, the degree of this activation appears to partially reverse, at the single cell level.

(iii) TNFα primes the superoxide response to fMLP after an optimal 30 min pre-incubation with human neutrophils. Although there is marked variability in the subsequent reversal of this enhancement, a residual priming effect is always apparent at 150 min.

6.2.2 Manipulation of the Priming Effects of TNFα

The principal aim of this Chapter was to establish whether sustained, receptor-mediated priming effects could be manipulated and reversed. However, the unexpected variability in the duration of the superoxide-priming effects of TNFα, together with the finding that neutrophils could remain polarized whilst de-priming with respect to respiratory burst activity, confounded the subsequent manipulation of these responses. Nevertheless, we decided to continue with our investigations, in the knowledge that the neutrophils of approximately 50% of donors showed no significant reduction in their superoxide-priming effect within the 2 hour period (Figure 6.4). Two strategies were chosen to evaluate the contribution of sustained TNFα receptor occupancy to the maintenance of the primed state. Since it was suggested that the extracellular ligand concentration (and hence the magnitude of the initial and ongoing response) might determine the rate of neutrophil de-priming (Chapter 5), the reversibility of priming induced by different concentrations of TNFα was first investigated.

6.2.2.1 The Influence of Ligand Concentration on the Reversibility of TNFα-Induced Neutrophil Priming

Human neutrophils were preincubated for 2 hours with three different concentrations of TNFα. The selected TNFα concentrations were: (i) the optimal priming concentration of 100 U/ml; (ii) a sub-maximal concentration of 20 U/ml, approximating the IC_{50} value for priming of the fMLP-superoxide anion response; and (iii) a supra-maximal concentration of 300 U/ml. A 30 min preincubation period with each of these concentrations was optimal for the priming of fMLP-stimulated superoxide anion release (data not shown). As expected from the concentration-dependency of these priming effects (Figure 6.3), 100 U/ml TNFα elicited the greatest degree of priming, whilst 20 U/ml TNFα elicited the least. However, when these different priming potentials were accounted for, there was no significant

difference in the rate of de-priming between the three ligand concentrations (Figure 6.5). Furthermore, within 90 min of TNFα exposure, the superoxide priming effect had reached a level that was similar for all three TNFα concentrations, and remained so for a further 30 min period.

6.2.2.2 The Differential Effects of Anti-TNF-R55 and Anti-TNF-R75 Antibodies on TNFα-Induced Priming of Superoxide Anion Release

Since the concentration of TNFα (20-300 U/ml) was found to affect neither the rate nor the extent of spontaneous reversal of the superoxide-priming effect, the contribution of each TNF-R subtype to the maintenance of the primed response was next investigated. Two, specific, mouse monoclonal, antagonistic antibodies (mAb) were utilized: (i) an IgG_1 anti-human TNF-R55 mAb, and (ii) an IgG_{2A} anti-human TNF-R75 mAb.

Preliminary flow-cytometric (antibody titration) analysis demonstrated that the neutrophil binding of each antibody reached a plateau at approximately 28 μg/ml. Furthermore, a 30 min pre-incubation with either anti-TNF-R55 (28 μg/ml) or anti-TNF-R75 (28 μg/ml) was shown to cause optimal inhibition of the early pro-apoptotic effect of TNFα (250 U/ml) in cultured human neutrophils (Joanna Murray, personal communication): control antibodies (isotype-matched, anti-human IL-2-R α-chain) failed to affect this response, and neither anti-TNF-R mAb affected the basal rate of neutrophil apoptosis following 6 or 20 hours in culture.

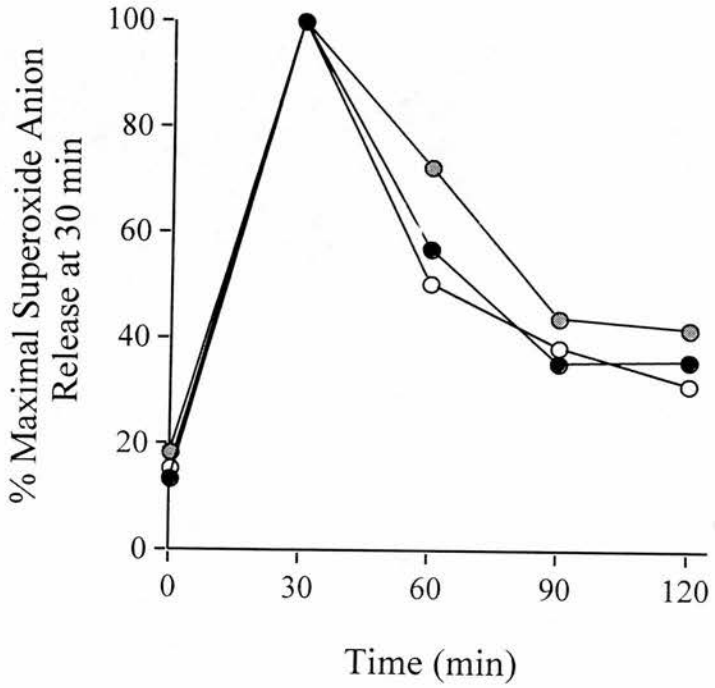


Figure 6.5

Effect of Ligand Concentration on the Time-Course for TNFα-Induced Priming of fMLP-Stimulated Superoxide Anion Release.

Neutrophils were incubated for 0-120 min with 100 U/ml TNFα (black circles), 20 U/ml TNFα (grey circles) or 300 U/ml TNFα (white circles). Following treatment with fMLP (100 nM, 10 min) in the presence of 1 mg/ml cytochrome C, superoxide anion release was assessed by scanning spectrophotometry. Values are expressed as % maximal superoxide anion release at 30 min for each TNFα concentration (100% values (nmol/10^6 cells): 100 U/ml TNFα 22.7 ± 3.2; 20 U/ml 15.3 ± 2.7; 300 U/ml 20.8 ± 2.9. Mean, n = 4 in duplicate. SEM values all <10% of mean and are omitted for reasons of clarity).

Human neutrophils were pre-incubated for 30 min with anti-TNF-R55 (28 μg/ml), anti-TNF-R75 (28 μg/ml), or both antibodies together, in order to establish the contribution of each TNF-R subtype to the priming effects of TNFα. After a 30 min pre-incubation, neither antibody alone nor in combination had any significant effect on the superoxide responses of control or 100 nM fMLP-stimulated neutrophils (Figure 6.6). However, anti-TNF-R55 mAb markedly inhibited (86 ± 6%) the TNFα-primed superoxide response; anti-TNF-R75 mAb had a far smaller inibitory effect (8 ± 5%). When both antibodies were used together, the priming effect of TNFα was effectively abolished (96 ± 3% inhibition). Following an extended 2 hour mAb pre-incubation with human neutrophils, these inhibitory actions persisted. These findings imply that TNF-R55 is the principal TNF-R subtype mediating the superoxide-priming effects of TNFα in suspension neutrophils whilst TNF-R75 plays a very minor role.

6.2.2.3 The Potential for TNF-R55 and TNF-R75 Blockade to Reverse TNFα-Induced Priming of Superoxide Anion Release

Having established the contribution of each TNF-R subtype to the initiation of TNFα-mediated priming of superoxide anion release, we next investigated the role of TNF-R55 and TNF-R75 in the maintenance of the primed state. Therefore, anti-TNF-R55 mAb (28 μg/ml), anti-TNF-R75 mAb (28 μg/ml) or both antibodies together, were introduced into the neutrophil incubation 30 min after the addition of TNFα (100 U/ml). A 90 min incubation with anti-TNF-R55 mAb caused a significant reduction (76 ± 6%) in the residual TNFα-mediated priming of fMLP-stimulated superoxide anion release (Figure 6.7); a smaller reduction was observed with anti-TNF-R75 mAb (44 ± 5%). When both TNF-R55 and TNF-R75 mAbs were combined, the reduction was approximately mid-way (60 ± 7%) between the individual effects of the two antibodies. Although investigations were hampered by the spontaneous reversibility of the TNFα-mediated superoxide-priming effect, these data support a predominant role for TNF-R55 in the maintenance of the primed state.

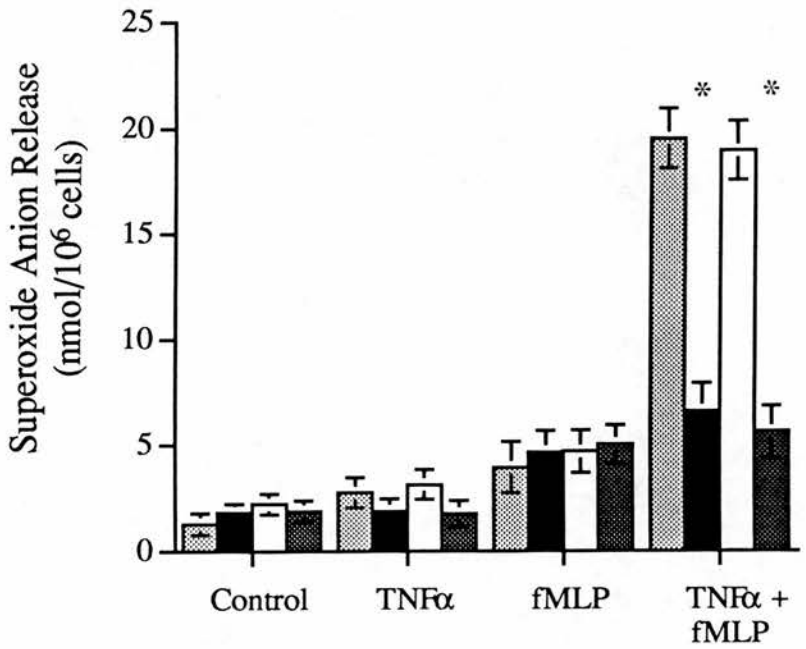


Figure 6.6
Effect of TNF-R55 and TNF-R75 Blockade on TNFα-Induced Priming of fMLP-Stimulated Superoxide Anion Release.
Neutrophils were pre-incubated for 30 min with anti-TNF-R55 mAb (28 μg/ml, black bars), anti-TNF-R75 mAb (28 μg/ml, white bars), anti-TNF-R55 plus anti-TNF-R75 mAbs (dark grey bars), or buffer (light grey bars). Following treatment with TNFα (100 U/ml, 30 min) or buffer control, neutrophils were incubated with fMLP (100 nM, 10 min) or buffer in the presence of 1 mg/ml cytochrome C. Superoxide anion release was assessed by scanning spectrophotometry (mean ± SEM, n = 4 in duplicate). *P<0.05, significantly different from values obtained in the absence of antibody (ANOVA).

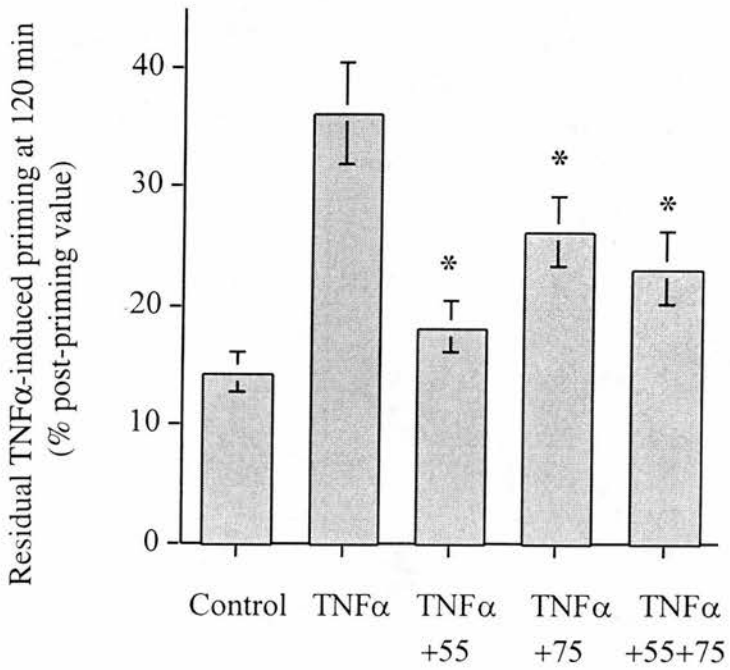


Figure 6.7

Effect of TNF-R55 and TNF-R75 Blockade on the Reversibility of TNFα-Induced Priming of fMLP-Stimulated Superoxide Anion Release.

Neutrophils were incubated for 30 min with 100 U/ml TNFα (represents "post-priming value") or PBS control prior to a further 90 min incubation in the presence of anti-TNF-R55 mAb (28 μg/ml, 55), anti-TNF-R75 mAb (28 μg/ml, 75), anti-TNF-R55 plus anti-TNF-R75 mAbs (55 + 75) or buffer control. Neutrophils were then incubated with fMLP (100 nM, 10 min) and superoxide anion release was assessed spectrophotometrically. Data represent percent of post-priming value remaining at 120 min (100% value = 22.6 ± 3.4 nmol $O_2^-/10^6$ cells). Mean ± SEM, n = 4 in duplicate. *$P<0.05$, significantly different from values obtained in the absence of antibody (ANOVA).

6.2.2.4 The Differential Effects of Anti-TNF-R55 and Anti-TNF-R75 Antibodies on TNFα-Induced Neutrophil Shape Change

Owing to the limitations incurred by the spontaneous reversal of TNFα-mediated priming of superoxide anion release, the capacity to reverse the associated, persistent shape change response was next investigated. In order to establish the contribution of each TNF-R subtype to the initiation of the polarization response, human neutrophils were pre-incubated for 30 min with anti-TNF-R55 mAb (28 μg/ml), anti-TNF-R75 mAb (28 μg/ml) or both antibodies together, before the addition of 100 U/ml TNFα or buffer for 30 min. Neither antibody had any effect upon the resting morphology of control neutrophils (Figure 6.8). However, while anti-TNF-R55 mAb could markedly inhibit (78 ± 7%) the shape change elicited by TNFα, anti-TNF-R75 had no inhibitory effect (11 ± 2% enhancement). When the two antibodies were used in combination, no augmentation of the action of anti-TNF-R55 mAb was observed (70 ± 6% inhibition). Thus, it would appear that TNF-R55 is also the principal TNF-R subtype through which TNFα mediates the shape change of human neutrophils.

6.2.2.5 The Influence of TNF-R55 Blockade on the Reversibility of TNFα-Induced Neutrophil Shape Change

The demonstration that TNF-R55 was the predominant TNF-R subtype involved in the induction of shape change by TNFα suggested that the subsequent blockade of this receptor might promote the reversal of the sustained polarization response. Therefore, human neutrophils were primed with TNFα under optimal conditions (100 U/ml, 30 min) prior to incubation with anti-TNF-R55 mAb (28 μg/ml) for a further 90 min. However, to our surprise, the TNFα-induced polarization response was not diminished (9 ± 2% enhancement) by the inclusion of anti-TNF-R55 mAb (Figure 6.9) (there was also a small increase (15 ± 4%) in the shape change of control neutrophils following antibody treatment). These observations imply that the maintenance of TNFα-induced neutrophil shape change does not require sustained activation of TNF-R55 beyond 30 min.

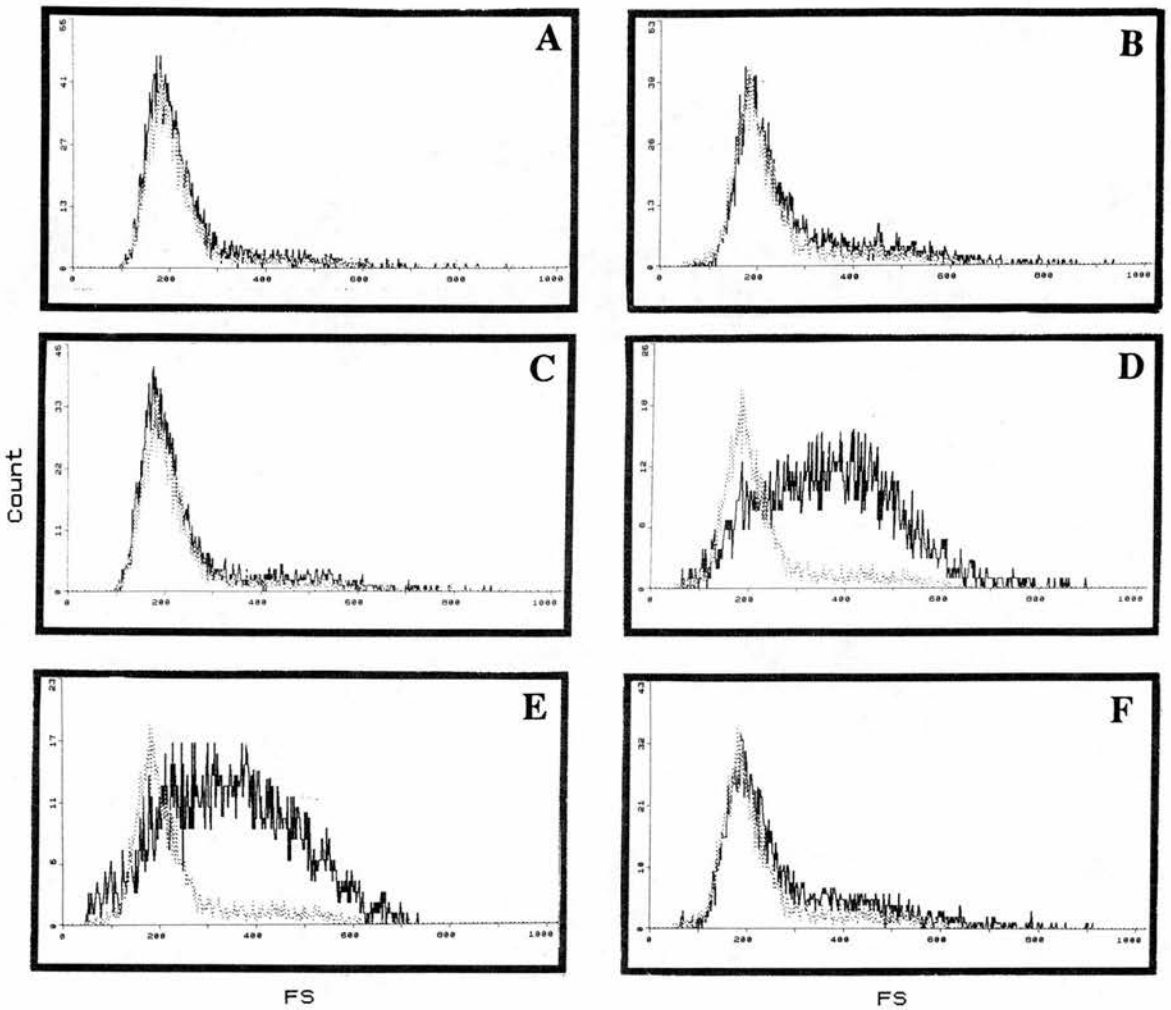


Figure 6.8
Effect of TNF-R55 and TNF-R75 Blockade on TNFα-Induced Neutrophil Shape Change.
Neutrophils were pre-incubated with anti-TNF-R55 mAb (28 μg/ml, A and B), anti-TNF-R75 mAb (28 μg/ml, C and D), anti-TNF-R55 plus anti-TNF-R75 mAbs (F) or buffer (E) for 30 min. Following treatment with TNFα (100 U/ml, 30 min, B, D, E and F) or buffer control (A and C), samples (black outlines) were analyzed for percent shape change and plotted against control samples (light grey outlines) (x-axis: mean forward light scatter, FS; y-axis: relative cell number). Representative flow-cytometry (EPICS Profile II) histograms (n = 4 in duplicate).

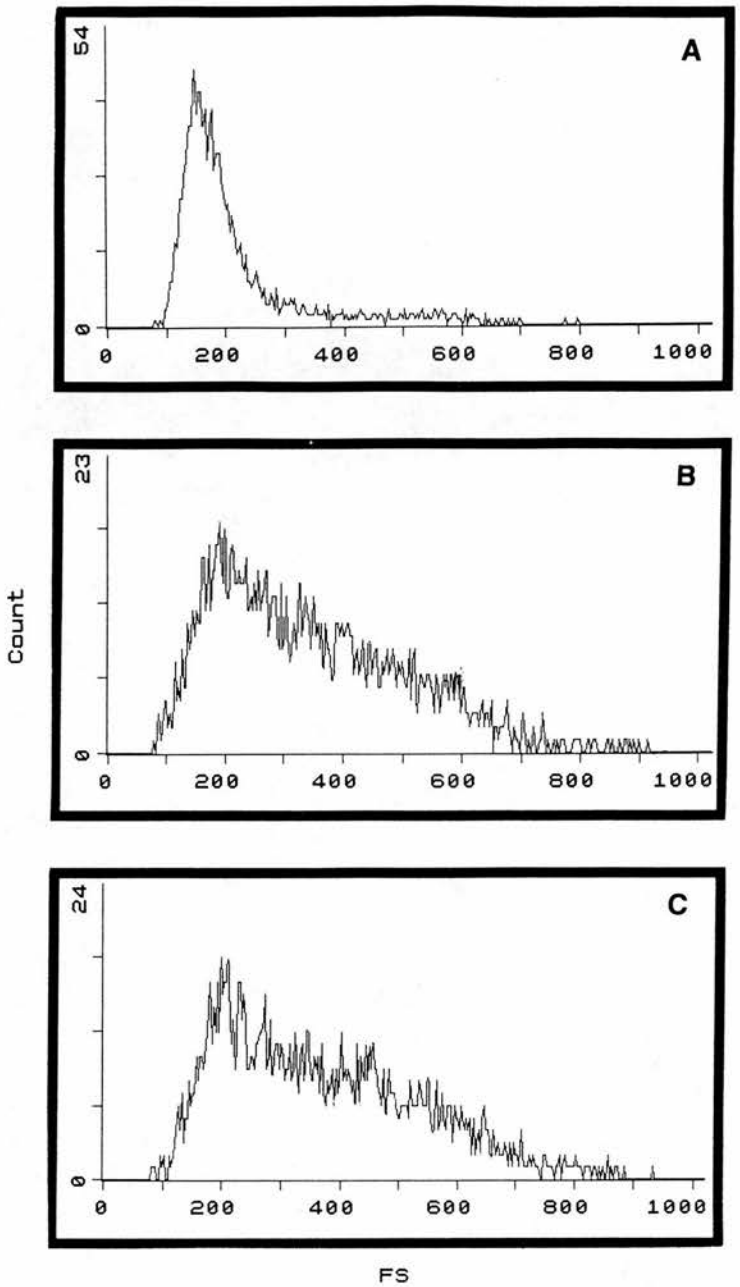


Figure 6.9

Effect of TNF-R55 Blockade on the Reversibility of TNFα-Induced Neutrophil Shape Change.

Neutrophils were incubated for 30 min with TNFα (100 U/ml, B and C) or buffer (A), prior to a further 90 min incubation in the presence of anti-TNF-R55 mAb (28 μg/ml, C) or buffer (A and B). Samples were analyzed for percent shape change by flow cytometry (x-axis: mean forward light scatter, FS; y-axis: relative cell number). Representative flow-cytometry (EPICS Profile II) histograms (n = 3 in duplicate).

6.3 Discussion

TNFα is an established, receptor-mediated priming agent that has been reported to have prolonged effects in human neutrophils (Shimizu *et al.*, 1993; Ozaki *et al.*, 1988; Ferrante *et al.*, 1988). Thus, it was selected as a model to investigate whether the duration of priming could be limited by the modulation of receptor occupancy. The responses elicited by TNFα were much slower in onset than those induced by PAF (Chapter 4), in agreement with our previous hypothesis that the time required to establish neutrophil priming may determine its subsequent reversibility. However, considerable differences were observed in the potential for each of the three indices of TNFα-mediated neutrophil priming to reverse. Indeed, a distinct and unique pattern of TNFα-mediated priming emerged, that is outlined below.

The shape change induced by TNFα was sustained, showing no significant reversal during the 2 hour period. This persistent polarization response confirms a previous observation (Shimizu *et al.*, 1993). Although the rapid and transient nature of PAF-induced neutrophil shape change (Chapter 4) suggested that this might be the earliest (and possibly the most sensitive) indicator of whether neutrophil priming would reverse spontaneously, the shape change response elicited by TNFα was the most stable of the three tested indices of priming. Thus, the relative duration of different priming responses may be agonist-specific.

TNFα elicited the functional upregulation of CD11b/CD18 on the surface of human neutrophils. When this response was quantified by sub-dividing neutrophils into those with or without attached fluorescent beads, the number of bead-binding neutrophils was maintained for at least 2 hours. This observation concurs with the recent report that TNFα causes a sustained increase in the surface expression of these adhesion molecules (Condliffe *et al.*, 1996). However, the sub-division of neutrophils into bead-binding or non-bead-binding populations takes no account of the actual number of beads bound to an individual neutrophil. Indeed, when

neutrophils were categorized with respect to the extent of bead-binding, the activation of CD11b/CD18 at the single cell level was seen to diminish after 60 min. This implies that TNFα-induced upregulation of CD11b/CD18 affinity or avidity (following integrin clustering) is more transient than either the increased expression of CD11b/CD18 or the accompanying neutrophil shape change.

In marked contrast to TNFα-induced shape change and the functional upregulation of CD11b/CD18, the duration of priming for enhanced superoxide anion release was highly variable. The rate of de-priming following TNFα treatment showed no correlation with the basal level of neutrophil priming, the concentration of TNFα, or the extent of TNFα-induced priming after 30 min, implying that such variability may be donor-dependent. In approximately 50% of cases there was no significant reduction in the enhancement of fMLP-stimulated superoxide anion release within the 2 hour period, confirming the prolonged priming effects of TNFα reported previously (Ozaki *et al.,* 1988; Atkinson *et al.,* 1988; Ferrante *et al.,* 1988). Although the remaining cases showed variable degrees of spontaneous reversibility, a residual priming effect of TNFα was always present at the end of the incubation: upon re-examination of the available literature, this reversibility was similar to that reported for the priming of fMLP-induced intracellular respiratory burst activity by 100 U/ml TNFα, where a residual (30% of maximal) priming effect was still present after 2 hours (Roberts *et al.,* 1993). However, a different group of investigators found that the sub-population of neutrophils primed by 100 U/ml TNFα had completely disappeared within 60 min (Elbim *et al.,* 1994), suggesting that neutrophils may have the potential to recover following TNFα-induced priming. Although different neutrophil isolation and incubation procedures may underlie such discrepancies between studies, it is clearly possible that the presentation of mean values may have masked genuine inter-donor variability in the capacity of neutrophils to de-prime following TNFα exposure.

Minimally primed human neutrophils that have been freshly isolated by Plasma-percoll separation express approximately equal numbers of the two TNF-R subtypes (Sarah Dunkley and Joanna Murray, personal communication). However, it is currently believed that TNF-R55 is the principal mediator of the bioactivities of TNFα in all cell types, including neutrophils (Tartaglia and Goeddel, 1992; Barbara *et al.,* 1994; Loetscher *et al.,* 1993; Espevik *et al.,* 1990). In agreement with this, both the induction of neutrophil shape change by TNFα, and the priming of fMLP-stimulated superoxide anion release were found to be almost exclusively dependent upon the activation of TNF-R55. This confirms a previous report where agonistic anti-TNF-R55 antibodies were shown to upregulate the surface expression of CD11b/CD18 on human neutrophils, and to enhance fMLP-stimulated release of superoxide anions, elastase and lactoferrin (Abe *et al.,* 1995). Whilst agonistic anti-TNF-R75 antibodies were without effect in this latter study, the use of selective, mutein (mutant protein) receptor agonists has implicated an accessory role for TNF-R75 in the priming of superoxide anion release (Barbara *et al.,* 1994). Our own investigations suggest that: (i) any co-operative effect of TNF-R75 with TNF-R55 for the priming of superoxide anion release is very modest; and (ii) TNF-R75 plays no part in (or even inhibits) the polarization response to TNFα.

In Chapter 5, it was proposed that the primed neutrophil state would be maintained so long as stimulatory signals were received by the cell. This was based upon the demonstration that neutrophil polarization to both C5a and fMLP is sustained until the cells are washed (Smith *et al.,* 1979), and the notion that the transient priming effects of PAF may be secondary to PAF receptor desensitization and the rapid internalization of this ligand by neutrophils (Bratton *et al.,* 1992; O'Flaherty *et al.,* 1986). However, following incubation with TNFα, there is a rapid and dramatic reduction (at least 60% by 60 min) in the surface expression of both TNF-R55 and TNF-R75 (Sarah Dunkley, personal communication). The almost instantaneous down-regulation observed in TNF-R55 may be secondary to its rapid internalization (maximal within 5 min) (Mosselmans *et al.,* 1988); although TNF-R75 lacks the tyrosine consensus sequence necessary for internalization (Collawn *et al.* 1990), both

TNF-75R and TNF-R55 can be shed from the surface of stimulated neutrophils (Porteu and Hieblot, 1994; Lantz *et al.,* 1990; Schleiffenbaum and Fehr, 1990; Porteu and Nathan, 1990). These proteolytically cleaved TNF-Rs were initially identified as soluble TNFα-binding proteins in serum (Peetre *et al.,* 1988; Gatanaga *et al.,* 1990), and may serve to dampen the effects of TNFα by sequestering it from membrane-bound receptors or, at lower concentrations, prolong the effects of TNFα by serving as a reservoir of biologically-active TNFα (Aderka *et al.,* 1992).

Despite the rapid down-regulation of both TNF-R subtypes that occurs in the presence of TNFα, neutrophils remained polarized for at least 2 hours following TNFα treatment. Furthermore, optimally-established shape change could not be reversed by the selective blockade of TNF-R55 (the dominant, if not exclusive, receptor subtype involved in the initiation of neutrophil polarization to TNFα). These observations imply that persistence of TNFα-mediated shape change is not dependent upon the continual stimulation of neutrophils through surface TNF-Rs.

In contrast, the residual primed state of respiratory burst activity observed 2 hours after the addition of TNFα does appear to depend upon the ongoing activation of TNF-R55 by TNFα. Although these investigations were hampered by the spontaneous reversal of the TNFα-induced priming effect, they suggest that stimulation of the small population of TNF-Rs remaining at the cell surface may be sufficient to maintain a primed state of oxidase activity. However, TNF-R75 appeared to hinder, rather than aid, the function of TNF-R55 in the maintenance of the superoxide priming effect: this might be secondary to the re-expression of TNF-R75, but not TNF-R55, which begins approximately 2-3 hours following TNFα treatment (Sarah Dunkley, personal communication). Consequently, the higher affinity of TNF-R75 for TNFα (Schall *et al.,* 1990; Loetscher *et al.,* 1990) may sequester ligand from TNF-R55, arguing against a ligand passing model. On the contrary, agonistic anti-TNF-R75 and anti-TNF-R55 antibodies have been shown to act synergistically to maintain CD11b/CD18 expression and neutrophil adhesion

following a 2-3 hour exposure to TNFα (Abe *et al.*, 1995). Thus, any interaction between the two TNF-R subtypes may depend upon the functional response.

In conclusion, it appears that the inherent reversibility of TNFα-mediated priming of superoxide anion generation may depend on negative feedback of intracellular events, as proposed previously for PAF (Chapter 5). The rate of onset and efficiency of the various feedback steps may underlie the differences between the duration of: (i) the three indices of priming; and (ii) PAF- and TNFα-primed responses. However, maintenance of the superoxide priming effect (and possibly CD11b/CD18 avidity) may also rely upon the availability of functional TNF-R (especially TNF-R55) and therefore, ultimately, on the combined rate of TNF-R internalization and shedding. Since TNF-R expression can be influenced by various pro-inflammatory stimuli (Porteu and Nathan, 1990), the pre-stimulation of neutrophils *in vivo* may also contribute to the subsequent *in vitro* variability in the superoxide priming effects of TNFα. Thus, the regulation of receptor expression may provide an effective means of limiting certain neutrophil responses to TNFα. Finally, if inter-donor variability does exist in the capacity of neutrophils to maintain a primed response to TNFα, this may reflect important differences in TNF-R function and regulation between subjects, and therefore merits further investigation.

7. CHAPTER 7: SUMMARY

The work in this thesis has addressed the potential for neutrophil priming to reverse. It was essential to commence with a population of pure yet minimally-primed human neutrophils in order to avoid the restrictions engendered by basally-primed control responses. Thus, peripheral blood neutrophils were isolated using dextran sedimentation and plasma/Percoll gradients, with meticulous attention paid to the maintenance of a sterile "LPS-free" environment at all times. The continual maintenance of incubation temperature and neutrophil suspension allowed prolonged *in vitro* investigations to be performed using "quiescent" and viable neutrophils.

The priming effects of certain pro-inflammatory agents (e.g. LPS, GM-CSF, G-CSF and IFN-γ) have been reported to persist for several hours. However, the observation that physico-chemical stimuli (such as hypotonic shock and cell swelling) could prime neutrophils in a reversible manner suggested that priming may not always be sustained. To examine the possible *in vivo* significance of this reversible priming process, we initially selected $InsP_6$ as a potential priming agent present at high micromolar concentrations in the cytoplasm of cells (and hence likely to be released from effete cells at inflammatory foci). Although the previously reported *in vitro* priming effect of $InsP_6$ (100 μM) was confirmed and found to be rapid (30-120 s), the magnitude of the priming effect was slight in comparison to LPS and was not mediated by specific extracellular receptors. Thus, an alternative mechanism, such as the cation-chelating properties of $InsP_6$, is proposed to underlie the observed priming ability. Nevertheless, the capacity of $InsP_6$ to induce membrane perturbations may link the transient priming effects of this agent, hypotonic shock and cell swelling.

An alternative approach for examining potential neutrophil de-priming was then pursued using PAF, a well-established, receptor-mediated priming agent. PAF (1 μM) was shown to act rapidly (≤10 min), eliciting considerable enhancement of fMLP-stimulated respiratory burst activity (the gold standard indicator of priming),

whilst inducing cell polarization and the functional up-regulation of CD11b/CD18. However, these priming-associated effects were not sustained, but underwent spontaneous and complete reversal within 2 hours: neutrophils remained viable and fully responsive to PMA throughout this time. Neither the release of adenosine (a paracrine inhibitor of neutrophil responses) nor the metabolism of PAF appeared to be involved in the reversal of the priming effects of PAF. On the contrary, the specific blockade of PAF receptors with WEB 2086 and UK-74,505 (added 10 min after PAF) increased the rate of decay of the PAF-primed superoxide response, suggesting that PAF receptor desensitization was not complete 10 min after PAF treatment. Since PAF-primed neutrophils that had spontaneously recovered could subsequently be fully re-primed when challenged with either PAF or TNFα, this represented the first demonstration that priming in response to a receptor-mediated agent was fully reversible.

We then investigated whether the receptor-mediated priming effects of TNFα, which had previously been reported to be more sustained than those induced by PAF, could be artificially manipulated and reversed. Optimal neutrophil priming by TNFα (100 U/ml) required a 30 min incubation and was shown to be mediated predominantly through the TNF-R55 subtype. Whilst the TNFα-induced polarization response was maintained for at least 2 hours, the accompanying functional up-regulation of CD11b/CD18 showed signs of decay (at the single cell level) within this time period, and the duration of priming for fMLP-stimulated superoxide anion release varied dramatically between experiments. Since inter-donor variability is inherent to many studies of neutrophil activation, it may have been responsible for the latter effect of TNFα; however, the pronounced variability in the priming effects of TNFα is in marked contrast to the reproducible effects of PAF, $InsP_6$ or hypotonic shock, and suggests that the mechanism of priming induced by TNFα is more complex than that elicited by these other agents.

From the data presented in this thesis and other published reports, it would appear that neutrophil priming *in vitro* may fall into two distinct categories: (1) fully

reversible (e.g. that induced by PAF, hypotonic shock, cell swelling, $InsP_6$ and potentially IL-8); or (2) largely irreversible (e.g. with GM-CSF, G-CSF, IFNγ and LPS). Since the boundaries between these two groups are indistinct, further study is required to classify agents such as TNFα. However, in general, agents in group (1) require shorter incubation periods to elicit their optimal priming effects than group (2) members. Thus, the rate of onset of neutrophil priming may be a key determinant of its subsequent reversibility. In the absence of any clear mechanistic basis for neutrophil priming, it is possible that the discrete signalling pathways used by individual priming agents will ultimately dictate both the speed and duration of the elicited responses. Nevertheless, it is also possible that neutrophils, given sufficient time, may eventually recover from all types of priming: their inherently short life-span dictates whether recovery will be detected.

It is unlikely that neutrophils within an inflammatory focus would be exposed in isolation to PAF or indeed other "transient" priming agents. It is more plausible that a number of pro-inflammatory mediators act in synergy to allow fine-tuning of the overall inflammatory response, thereby defining the exact balance between the microbicidal and potentially tissue-damaging consequences of neutrophil activation. However, individual agents clearly have a characteristic pattern of priming responses *in vitro* which probably reflects their functional roles *in vivo*. One might speculate that priming agents with a long duration of action may be responsible for maintenance of functionally-upregulated neutrophils following widespread or prolonged inflammatory insults; priming agents with transient effects may be more important in the early stages of the acute inflammatory response. In this model, a persistent state of neutrophil hyper-responsiveness following minimal focal trauma would be more detrimental than beneficial to the host. Since neutrophil priming is becoming increasingly associated with a variety of pathological states such as ARDS and rheumatoid arthritis, the recognition that neutrophils have the potential to de-prime may allow the pro-inflammatory effects of neutrophil priming/activation to be counteracted in its very earliest stages.

8. CHAPTER 8: BIBLIOGRAPHY

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9. CHAPTER 9: PUBLICATIONS

9.1 PAPERS

Kitchen, E., Condliffe, A.M., Rossi, A.G., Haslett, C., Chilvers, E.R. (1996) Characterization of inositol hexakisphosphate ($InsP_6$)-mediated priming in human neutrophils: lack of extracellular [^{3}H]-$InsP_6$ receptors. *Brit. J. Pharmacol.* 117: 979.

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9.2 ABSTRACTS

Kitchen, E., Condliffe, A.M., Haslett, C., Chilvers, E.R. (1994) Identification of [^{3}H]inositol hexakisphosphate binding sites in human neutrophils. *Am. Rev. Respir. Dis.* 149: A361.

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Characterization of inositol hexakisphosphate ($InsP_6$)-mediated priming in human neutrophils: lack of extracellular [^{3}H]-$InsP_6$ receptors

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1 Inositol hexakisphosphate ($InsP_6$) is a ubiquitous and abundant cytosolic inositol phosphate that has been reported to prime human neutrophils for enhanced agonist-stimulated superoxide anion generation. This led to the proposal that the release of $InsP_6$ from necrotic cells may augment the functional responsiveness of neutrophils at an inflammatory focus. The aim of this study was to examine whether the functional effects of $InsP_6$ in neutrophils are receptor-mediated and establish the magnitude of this priming effect relative to other better characterized priming agents.

2 Analysis of [^{3}H]-$InsP_6$ binding to human neutrophil membranes in 20 mM Tris, 20 mM NaCl, 100 mM KCl, 5 mM EDTA (pH 7.7) buffer using 0.1 mg ml^{-1} membrane protein and 2.5 nM [^{3}H]-$InsP_6$ (90 min, 4°C), demonstrated specific low affinity [^{3}H]-$InsP_6$ binding that was non-saturable up to a radioligand concentration of 10 nM.

3 [^{3}H]-$InsP_6$ displacement by $InsP_6$ gave a Hill coefficient of 0.55 and best fitted a two-site logistic model (53% K_D 150 nM, 47% K_D 5 μM). [^{3}H]-$InsP_6$ binding also displayed low (3 fold) selectivity for $InsP_6$ over Ins(1,3,4,5,6)P_5.

4 The specific [^{3}H]-$InsP_6$ binding displayed a pH optimum of 8, was abolished by pre-boiling the membranes, and was enhanced by Ca^{2+}, Mg^{2+} and Na^+.

5 In incubations with intact neutrophils, where high levels of specific [^{3}H]-LTB_4 binding was observed, no [^{3}H]-$InsP_6$ binding could be identified.

6 Preincubation of neutrophils with 100 μM $InsP_6$ had no effect on resting cell morphology, but caused a minor and transient (maximal at 30 s) enhancement of (0.1 nM) fMLP-induced shape change (% cells shape changed: fMLP 53±3%, fMLP + $InsP_6$ 66±4%). Similarly, $InsP_6$ (100 μM, 30 s) had no effect on basal superoxide anion generation and, compared to lipopolysaccharide (LPS, 100 ng ml^{-1}, 60 min), tumour necrosis factor-α (TNFα, 200 u ml^{-1}, 30 min) or platelet-activating factor (PAF, 100 nM, 5 min) caused only a small enhancement of 100 nM fMLP-stimulated superoxide anion generation (fold-increase in superoxide anion generation over fMLP alone: $InsP_6$ 1.8±0.3, LPS 6.8±0.6, TNFα 5.2±0.7, PAF 5.8±0.6).

7 While these data support the presence of a specific, albeit low affinity, [^{3}H]-$InsP_6$ binding site in human neutrophil membrane preparations, the lack of binding to intact cells implies that the functional effects of $InsP_6$ (ie. enhanced fMLP-stimulated superoxide anion generation and shape change) are not receptor-mediated.

Keywords: Inflammation; neutrophil priming; inositol hexakisphosphate; superoxide anions; neutrophil shape-change

Introduction

Inositol hexakisphosphate ($InsP_6$) is the most abundant inositol phosphate found in nature (Cosgrove, 1980), being present in mammalian cells at concentrations between 10 μM and 1 mM (Szwergold *et al.*, 1987). It is an intriguing molecule, whose true physiological role has yet to be revealed. Intracellularly, $InsP_6$ has been proposed to function as a general antioxidant (Graf & Eaton, 1990), Ca^{2+} chelator (Luttrell, 1993), inhibitor of iron-catalysed hydroxyl radical formation (Hawkins *et al.*, 1993) and phosphate store (Berridge & Irvine, 1989). It is also a specific inhibitor of a number of the enzymes involved in inositol polyphosphate metabolism, for example the Ins(1,3,4,5)P_4 3-phosphatase (Hughes & Shears, 1990; Höer & Oberdisse, 1991), and can itself be metabolized into a series of more polar inositol polyphosphates termed pyrophosphates (Mennite *et al.*, 1993; Stephens 1993). Investigations into the effects of calcium-mobilizing agonists on cellular $InsP_6$ levels have demonstrated either no effect (Glennon & Shears, 1993), or a rapid, transient increase that parallels Ins(1,4,5)P_3 accumulation (Sasakawa *et al.*, 1993). In addition, quite marked changes in the concentrations of both $InsP_5$ and $InsP_6$ can be seen with progression through the cell cycle or changes in cell phenotype (e.g. during neutrophilic differentiation of HL-60 cells) (French *et al.*, 1991; Guse *et al.*, 1993).

There is growing evidence that $InsP_6$ may also have a number of extracellular actions. Initial interest focused on its ability to suppress the development of colonic cancer in animal models, probably by chelating metal ions and thereby limiting mitogenic iron-catalysed redox reactions (Graf & Eaton, 1993). It has also been shown to lower blood pressure and heart rate in a reversible manner when infused into specific regions of the rat brainstem (Vallejo *et al.*, 1987). At a cellular level, $InsP_6$ has been shown to elicit Ca^{2+} influx and catecholamine release in bovine adrenal chromaffin cells (Regunathan *et al.*, 1992) and to enhance Ca^{2+} influx in cultured neuronal cells (Nicoletti *et al.*, 1989). However, the powerful Ca^{2+} chelation (Cosgrove, 1980) and autofluorescence properties of $InsP_6$ complicate the interpretation of such studies (Sun *et al.*, 1992).

It has recently been reported that $InsP_6$ may also function as a neutrophil priming agent and hence have a pro-inflammatory role (Eggleton *et al.*, 1991). Preincubation of neutrophils with 10–250 μM $InsP_6$ was shown to enhance subsequent agonist-

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induced superoxide anion generation and result in a rapid and sustained assembly of F-actin (Crawford & Eggleton, 1992). This led to the proposal that release of $InsP_6$ from necrotic cells at an inflammatory focus may upregulate, or prime, the functional responsiveness of adjacent neutrophils to secretagogue agonists. Since priming has been shown to be a prerequisite for neutrophil-mediated tissue injury, this event could play a vital role in modulating the extent of inflammation-induced organ damage (Smedley *et al.*, 1986).

In view of recent reports identifying the presence of specific, high affinity [^{3}H]-$InsP_6$ receptors in the rat brain (Hawkins *et al.*, 1990), and their subsequent characterization as the α-subunits of the clathrin assembly protein AP-2 (Volgmaier *et al.*, 1992), we have examined whether the reported functional effects of $InsP_6$ in human neutrophils are mediated by similar receptors. Our findings indicate that while specific [^{3}H]-$InsP_6$ binding sites are present on neutrophil membranes, they do not display the characteristic high affinity and selective $InsP_6$ binding properties reported in other cell types, and more importantly, are not present on intact cells: hence it is unlikely that the functional effects of $InsP_6$ are receptor-mediated. A more complete re-evaluation of the functional effects of $InsP_6$ demonstrates that this molecule has only very modest and transient effects on human neutrophil function compared to more established priming agents.

Methods

Neutrophil preparation

Blood was taken from healthy adult volunteers, anticoagulated with 4 ml 3.8% sodium citrate 40 ml^{-1} blood, and centrifuged (300 *g*) for 20 min. Neutrophils were isolated as detailed by Haslett *et al.* (1985) using dextran sedimentation and discontinuous plasma-Percoll gradients. The purified neutrophils were washed sequentially in platelet-poor plasma, PBS without, and then PBS with Ca^{2+} and Mg^{2+}. All procedures were conducted at 25°C. Cell purity and viability (assessed by trypan blue exclusion), were routinely >95% (<0.5% monocyte contamination) and >99.5% respectively.

[^{3}H]-$InsP_6$ binding to neutrophil membranes

Membranes were prepared as detailed by Hawkins *et al.* (1990). In brief, neutrophils were resuspended at 15×10^6 cells ml^{-1} in 20 mM Tris (pH 7.7), 20 mM NaCl, 100 mM KCl and 5 mM EDTA (4°C), homogenized (Polytron), centrifuged (35,000 *g*, 30 min), and the resulting membranes washed twice before use. Membrane protein concentrations were determined by the Pierce-BCA protein assay with BSA as standard.

[^{3}H]-$InsP_6$ binding was performed according to the method of Hawkins *et al.* (1990). Freshly prepared membranes (0.1 mg ml^{-1}) were incubated at 4°C in 20 mM Tris (pH 7.7), 20 mM NaCl, 100 mM KCl and 5 mM EDTA with 2.5 nM (90,000 d.p.m.) [^{3}H]-$InsP_6$, in a final volume of 1 ml. Separation of bound from free radioligand was achieved by centrifugation (13,000 *g*, 6 min, 4°C), with non-specific binding (NSB) determined in the presence of 100 μM $InsP_6$. Pellets were dissolved overnight in Soluene and their radioactivity determined by liquid scintillation counting. In preliminary experiments, [^{3}H]-$InsP_6$ binding was found to be linear up to a protein concentration of 0.2 mg ml^{-1} with equilibrium between free and bound [^{3}H]-$InsP_6$ achieved by 90 min (data not shown).

To assess whether there was any metabolism of [^{3}H]-$InsP_6$ during these assays, pre- and post-incubation supernatants were analysed by anion exchange h.p.l.c., using a Partisphere 5-SAX column (250 × 4.6 mm) fitted with a Whatman SAX guard cartridge eluted (flow rate 1.25 ml min^{-1}, 0.3 min fractions) with the following gradient: A (H_2O), B (3.5 M ammonium formate, pH adjusted to 3.7 with orthophosphoric acid): 0–5 min 0% B; 10–12 min 21.4% B; 18–23 min 28.5% B; 30 min 40.0% B; 40 min 42.0% B; 60–65 min 100% B.

In competition assays, displacing agents ($InsP_6$, 0.1 nM–0.1 mM; Ins(1,3,4,5,6)P_5, 10 nM–0.1 mM and Ins(1,4,5)P_3, 10 nM–0.1 mM) were added in 100 μl (10× final concentrations) aliquots. The pH-dependency of [^{3}H]-$InsP_6$ binding was examined by resuspending the neutrophil membranes in 20 mM Tris, 20 mM NaCl, 100 mM KCl, 5 mM EDTA buffered over an appropriate pH range with Trizma maleate-HCl (pH 5.5–7.0) or Trizma base-HCl (7.5–9.0). The effect of the cations Mg^{2+} and Ca^{2+} on [^{3}H]-$InsP_6$ binding was investigated using predetermined EDTA, EGTA and $MgCl_2$ additions to the above buffer, as detailed in the results section. The effect of protein denaturation on [^{3}H]-$InsP_6$ binding was assessed by heating the membranes to 100°C for 90 min prior to use.

To examine whether the [^{3}H]-$InsP_6$ binding observed was to an intra- or extracellular site, assays were performed with intact, freshly prepared neutrophils (3×10^6 ml^{-1}, equivalent to 0.1 mg ml^{-1} protein) incubated at 4°C in either PBS containing 25 mM HEPES (pH 7.4) or 20 mM Tris (pH 7.5), 20 mM NaCl, 100 mM KCl and 5 mM EDTA. Cells were layered over 0.4 ml silicone oil, incubated for 90 min on ice and then centrifuged (15,000 *g*, 1 min). Aliquots (200 μl) of the supernatants were removed and transferred to scintillation vials. The remaining supernatant and oil layers were aspirated and discarded, and the cell pellets dissolved in methanol and radioactivity determined. Parallel incubations were performed to assess [^{3}H]-$InsP_6$ binding to neutrophil membranes prepared from the same batch of cells and [^{3}H]-LTB_4 binding to intact cells, as detailed previously (O'Flaherty *et al.*, 1986; 1991).

Neutrophil shape change assay

The effect of $InsP_6$ on fMLP-induced shape-change was assessed by incubating 3×10^6 neutrophils in 500 μl PBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 25 mM HEPES (pH 7.3) at 37°C, with a pre-determined optimal concentration of $InsP_6$ (100 μM), for 0.5–30 min prior to addition of 0.1 nM fMLP for 5 min. Preliminary concentration-response studies had identified this as the fMLP concentration required to induce submaximal (approx. 50%) shape change (data not shown). Incubations were terminated by the addition of 500 μl 2.5% gluteraldehyde and shape-change was quantified by phase contrast light microscopy as the percentage of neutrophils extruding more than one pseudopodium. Identical incubations were performed with LPS (100 ng ml^{-1}, 60 min), TNFα (200 u ml^{-1}, 30 min) and PAF (100 nM, 5 min).

Superoxide anion generation

Neutrophils were resuspended at 1×10^6 cells ml^{-1} in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 25 mM HEPES (pH 7.3) and preincubated at 37°C with buffer, $InsP_6$ (100 μM, 30 s), LPS (100 ng ml^{-1}, 60 min), TNFα (200 u ml^{-1}, 30 min) or PAF (100 nM, 5 min) in a final volume of 100 μl. These pretreatment periods and agonist concentrations were established in preliminary experiments designed to ascertain optimal priming conditions for each agent. The cells were then stimulated with fMLP (100 nM, 15 min) in the presence of 80 μM cytochrome C, with superoxide dismutase (375 u) added to one tube in each set of quadruplicate incubations. Reactions were terminated by placing the cells on ice followed by centrifugation (15,000 *g*, 5 min, 4°C). The superoxide-dismutase-inhibitable reduction of cytochrome C was determined in each supernatant by measurement of the peak absorbance between 535–565 nm, with a Pye-Unicam scanning spectrophotometer, and expressed as nmol superoxide anion generated per 10^6 cells.

Drugs and chemicals

Inositol hexakisphosphate ($InsP_6$, di-potassium salt), N-formyl-methionyl-leucyl-phenylalanine (fMLP), superoxide dismutase, cytochrome C, platelet-activating factor (PAF), lipopolysaccharide (LPS, E. coli 0111:B4), phosphate-buffered

saline (PBS, with or without $CaCl_2$ and $MgCl_2$), dextran-500 and Percoll were all purchased from Sigma (Poole). Tumour necrosis factor-α (TNFα) was obtained from Genzyme (Cambridge, MA, U.S.A.). Inositol pentakisphosphate ($Ins(1,3,4,5,6)P_5$) was purchased from Calbiochem (Nottingham) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) from RBI (St Albans). [^{3}H]-inositol hexakisphosphate (specific activity 15–24 Ci mmol^{-1}) was obtained from DuPont-New England Nuclear (Stevenage, Herts.). Silicone oil F-50 was obtained from Croylek Ltd. (Surrey). All other reagents and chemicals were purchased from Life Technologies (Paisley), BDH (Poole), Phoenix Pharmaceuticals Ltd. (Gloucester) or Packard (Pangbourne, Berks.) and were of the highest grade available.

Statistics

All values are expressed as means ± s.e.mean of (n) separate experiments. Values, where applicable, were compared by ANOVA or Student's t test for paired data, with $P<0.05$ considered to be significant. Significant differences between groups were determined by the Newman-Keuls procedure.

Results

[^{3}H]-$InsP_6$ binding sites in human neutrophil membranes

Under the assay conditions defined (2.5 nM [^{3}H]-$InsP_6$, 0.1 mg membrane protein, 90 min incubations on ice), total and non-specific [^{3}H]-$InsP_6$ binding represented approximately 3,000 (approximately 200 fmol mg^{-1} protein) and 300 d.p.m. respectively. Analysis of [^{3}H]-$InsP_6$ displacement by $InsP_6$ (Figure 1a) gave a Hill coefficient of 0.55 and a curvilinear bound versus bound × inhibitor plot (Figure 1b), indicating the presence of at least two binding sites. The curve was best-fitted to a two-site logistic model, where 53% of the $InsP_6$ bound to a site with a K_D of 150 nM and the remainder to a 5 μM K_D site. As predicted from these values, [^{3}H]-$InsP_6$ binding failed to saturate fully up to a radioligand concentration of 10 nM and kinetic experiments demonstrated incomplete displacement of steady-state [^{3}H]-$InsP_6$ binding following addition of 100 μM unlabelled $InsP_6$ (60% displacement at 45 min, data not shown). $Ins(1,3,4,5,6)P_5$ and $Ins(1,4,5)P_3$ displaced [^{3}H]-$InsP_6$ binding with IC_{50} values of 430 nM and 30 μM respectively ($n=8$) (Figure 1a). In the absence of membranes, total [^{3}H]-$InsP_6$ binding was equal to the non-specific binding determined in the presence of membranes. Incubations with pre-boiled membranes reduced specific [^{3}H]-$InsP_6$ binding by >90% ($n=8$, data not shown).

The possibility that the multi-site, low affinity [^{3}H]-$InsP_6$ binding observed was due to metabolism of the radioligand was investigated by h.p.l.c. analysis of the post-incubation supernatants, by a method designed to detect inositol hexakisphosphate metabolites ([^{3}H]-$InsP_{1\text{-}5}$) (Hawkins *et al.*, 1990). These experiments demonstrated a start radioligand purity of >99.9% and no detectable [^{3}H]-$InsP_6$ metabolism during the 90 min incubation period (data not shown).

Effect of pH on [^{3}H]-$InsP_6$ binding in human neutrophil membranes

Specific [^{3}H]-$InsP_6$ binding was markedly enhanced under alkaline conditions, with maximum binding at pH 8.0 (750 fmol mg^{-1} protein) (Figure 2). Non-specific binding was similar at all pH values studied (313 ± 24 d.p.m.).

Modulation of [^{3}H]-$InsP_6$ binding in human neutrophil membranes by mono- and divalent cations

In view of the suggestion that [^{3}H]-$InsP_6$ may associate with membranes through non-protein interactions, in a manner dependent upon trace metals (Poyner *et al.*, 1993), we examined the ability of various mono- and divalent cations to influence total [^{3}H]-$InsP_6$ binding to neutrophil membranes. For each buffer condition, [^{3}H]-$InsP_6$ binding was compared to that obtained in 20 mM Tris (pH 7.7), 20 mM NaCl, 100 mM KCl and 5 mM EDTA, with this value referred to as 100% binding (Figure 3). Omission of 5 mM EDTA increased total binding by 158 ± 20%. Replacement of the EDTA with 5 mM EGTA caused a 76 ± 25% increase in binding, with the further addition of 1 mM Mg^{2+} augmenting the binding by an additional 89 ± 3%. [^{3}H]-$InsP_6$ binding was also influenced by manipulating the concentration of Na^+ and K^+ present, with an increase in binding of 79 ± 18% seen in the absence of KCl and a decrease of 61 ± 6% seen with NaCl exclusion. Thus, the presence of Ca^{2+}, Mg^{2+} and Na^+ all appear to enhance, whereas K^+ inhibits, [^{3}H]-$InsP_6$ binding to human neutrophil membranes. Non-specific binding, determined in the presence of 100 μM $InsP_6$, was similar under all conditions studied

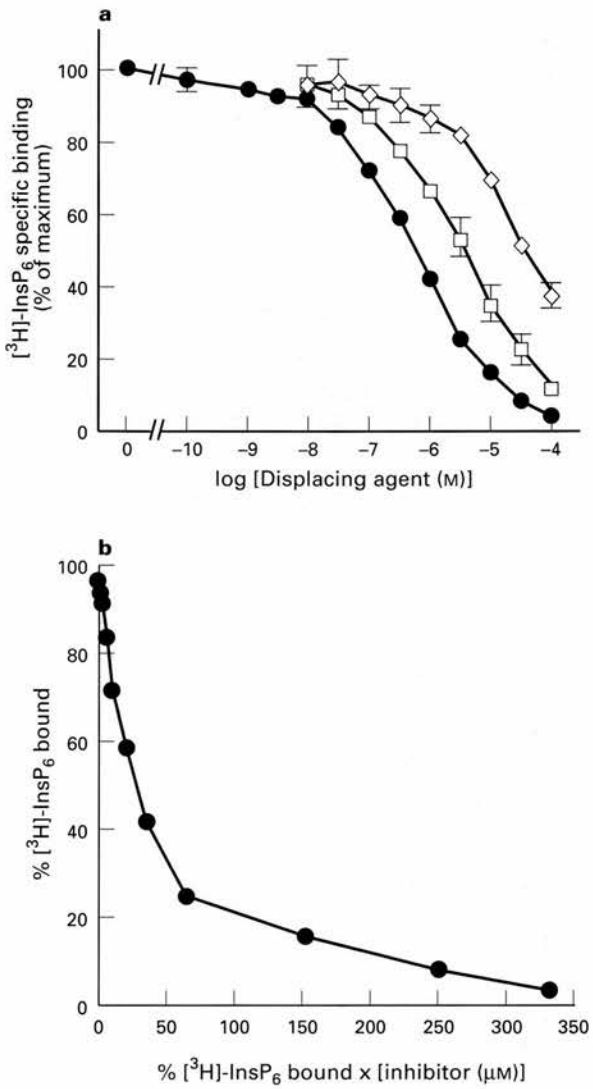

Figure 1 (a) Displacement of [^{3}H]-$InsP_6$ binding to human neutrophil membranes by $InsP_6$, $Ins(1,3,4,5,6)P_5$ and $Ins(1,4,5)P_3$; (b) bound versus bound × inhibitor plot for competition of [^{3}H]-$InsP_6$ binding by $InsP_6$. Assays were performed with 2.5 nM [^{3}H]-$InsP_6$, 0.1 mg of human neutrophil membrane fraction and increasing concentrations of $InsP_6$, (●), $Ins(1,3,4,5,6)P_5$ (□) and $Ins(1,4,5)P_3$ (◇) in 20 mM Tris/HCl/20 mM NaCl/100 mM KCl/5 mM EDTA buffer, pH 7.7 (final volume 1 ml). Incubations were performed for 90 min at 4°C, with separation of bound from free radioligand by centrifugation. Non-specific binding was determined in the presence of 100 μM unlabelled $InsP_6$. Values represent mean ± s.e.mean for 8 experiments each performed in duplicate.

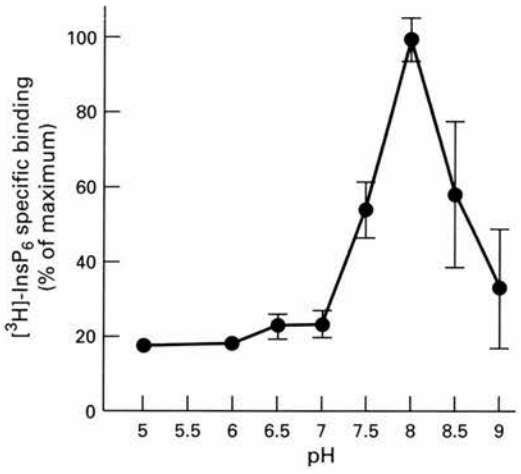

Figure 2 pH-dependence of specific [^{3}H]-$InsP_6$ binding to human neutrophil membranes. [^{3}H]-$InsP_6$ binding to human neutrophil membranes was determined using 2.5 nM [^{3}H]-$InsP_6$ and 0.1 mg membrane protein (as detailed in the legend to Figure 1) in a range of 25 mM Tris (pH 7.5–9) and Tris-maleate (pH 5.5–7) buffers (see Methods). Incubations were performed at 4°C for 90 min and non-specific binding determined in the presence of 100 μM unlabelled $InsP_6$. Values represent mean ± s.e.mean of maximal specific [^{3}H]-$InsP_6$ binding (13,355 ± 743 d.p.m.) for 6 determinations in two separate experiments.

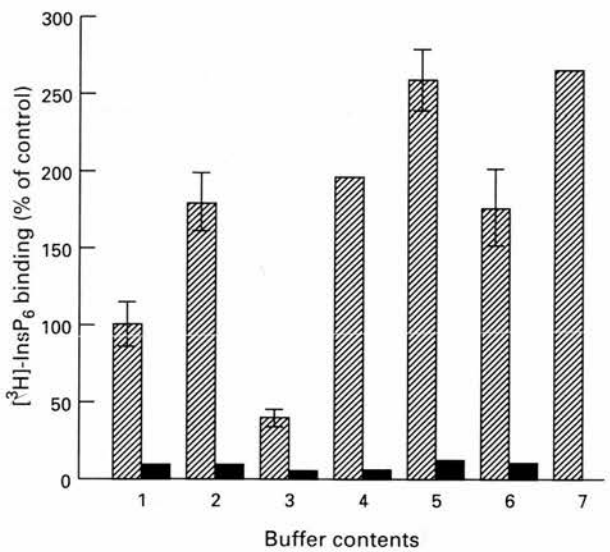

Figure 3 Effects of Mg^{2+}, Ca^{2+}, Na^+ and K^+ on [^{3}H]-$InsP_6$ binding to human neutrophil membranes. Assays were performed as outlined in the legend to Figure 1 except that following isolation, neutrophils were resuspended in a series of 20 mM Tris/HCl buffers (pH 7.7) with varying amounts of EDTA/EGTA/KCl/NaCl/$MgCl_2$ as detailed below. The cells were then homogenized, pelleted and resuspended in the same series of buffers at 0.1 mg protein ml^{-1} and [^{3}H]-$InsP_6$ binding (hatched columns) determined using 2.5 nM [^{3}H]-$InsP_6$ and an incubation period on ice of 90 min. Non-specific binding (solid columns) was determined in the presence of 100 μM unlabelled $InsP_6$. The buffers used were: Column (1), 5 mM EDTA, 100 mM KCl, 20 mM NaCl; Column (2), 5 mM EDTA, 20 mM NaCl; Column (3), 5 mM EDTA, 100 mM KCl; Column (4), 5 mM EDTA; Column (5), 100 mM KCl, 20 mM NaCl; Column (6), 5 mM EGTA, 100 mM KCl, 20 mM NaCl; Column (7), 5 mM EGTA, 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$. Values represent mean ± s.e.mean of 3 experiments each performed in duplicate. (Where not shown, s.e.means were < 2% of means and fall within symbols). 100% binding represents 4,958 ± 197 d.p.m. Non-specific binding in buffer 7 was > 70% total [^{3}H]-$InsP_6$ added (see Results).

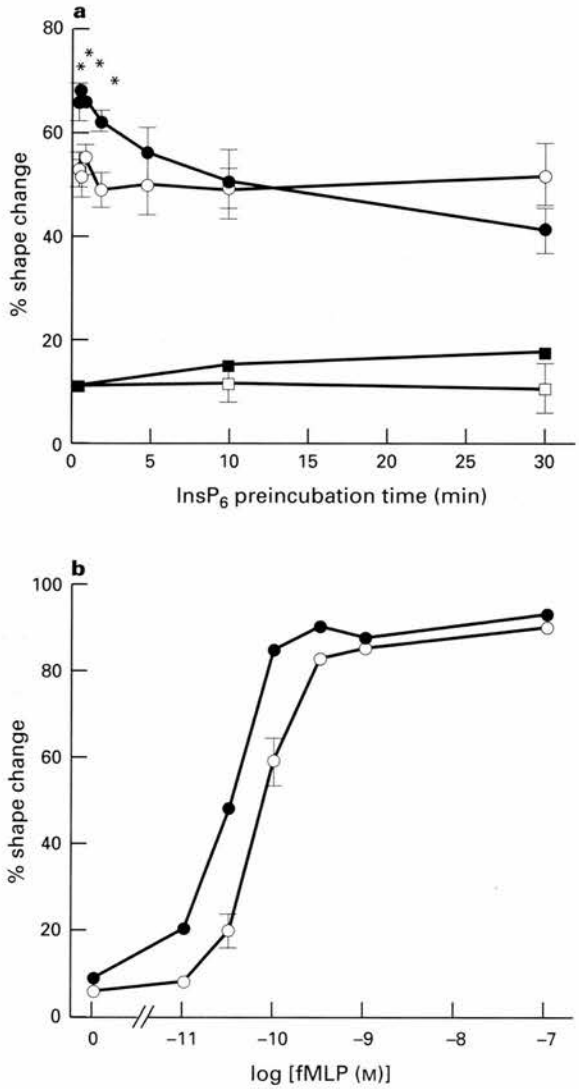

Figure 4 (a) Effect of $InsP_6$ preincubation time on fMLP-induced neutrophil shape change. Purified human neutrophils (3×10^6 ml^{-1}) were preincubated for various periods (0.5–30 min) with either $InsP_6$ (100 μM, closed symbols) or 20 mM HEPES PBS buffer (pH 7.3) (open symbols) prior to 5 min treatment with fMLP (0.1 nM, circles) or buffer (squares). Reactions were terminated, and shape change assessed as detailed in the Methods section. Values represent mean ± s.e.mean of 3 experiments, each performed in duplicate. $^*P < 0.05$, significantly different from fMLP alone (ANOVA). (b) Effect of $InsP_6$ on fMLP concentration-response curve for neutrophil shape change. Neutrophils were preincubated for 30 s with either $InsP_6$ (100 μM, closed symbols) or buffer (open symbols), prior to a 5 min treatment with fMLP. Values represent mean ± s.e.mean of triplicate determinations from a single experiment, with similar results obtained in a further 4 experiments.

(7.3 ± 0.8% of total binding) except that in the presence of 5 mM EGTA plus 1 mM $MgCl_2$ there was a dramatic increase in membrane pellet associated [^{3}H]-$InsP_6$ (52,014 ± 4,362 d.p.m. ie. approximately 70% of the total [^{3}H]-$InsP_6$ added), suggesting precipitation of an $InsP_6$-Mg^{2+} complex similar to that observed with Fe^{3+} concentrations > 10 μM (Poyner *et al.*, 1993).

[^{3}H]-$InsP_6$ binding to intact human neutrophils

A number of methods were used to assess whether the [^{3}H]-$InsP_6$ binding observed in neutrophil membranes represented binding to an intra- or extracellular recognition site. Incubation of freshly prepared neutrophils at 4°C for 90 min with

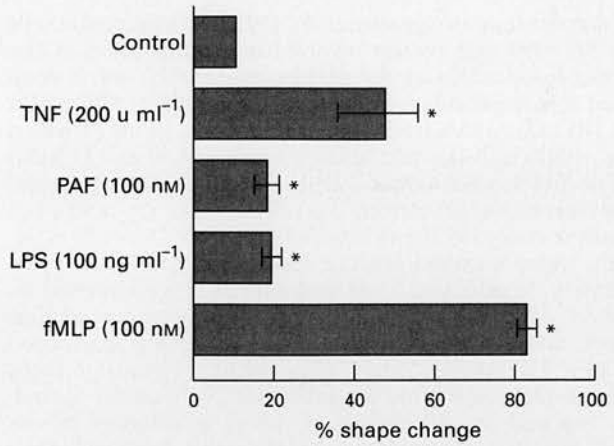

Figure 5 Effect of TNFα, PAF and LPS on neutrophil shape change. Human neutrophils (3×10^6 ml^{-1}) were incubated with TNFα (200 u ml^{-1}, 30 min), PAF (100 nM, 5 min), LPS (100 ng ml^{-1}, 60 min), fMLP (100 nM, 15 min), or 25 mM HEPES PBS buffer (pH 7.3) (control). Reactions were terminated, and shape change assessed as detailed in the Methods section. Values represent mean ± s.e.mean of 3 experiments, each performed in duplicate. Where not shown, s.e.means are < 2% of means and fall within symbols. $^*P < 0.05$ significantly different from control (ANOVA).

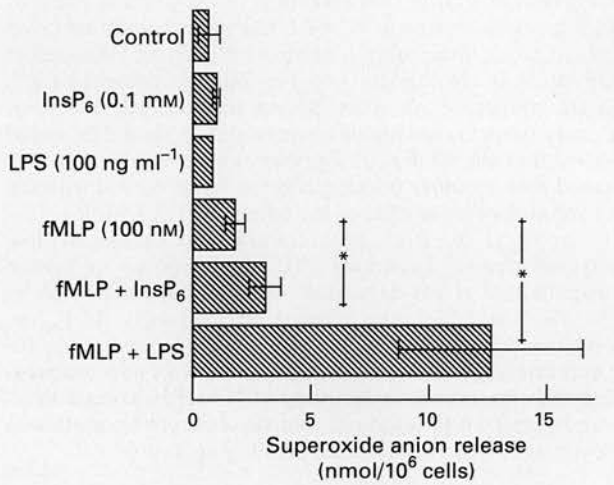

Figure 6 Comparison of the effects of $InsP_6$ and LPS on fMLP-induced superoxide anion generation in human neutrophils. Human neutrophils were suspended in PBS containing 25 mM HEPES as detailed in the Methods section and preincubated with 100 μM $InsP_6$ for 30 s or 100 ng ml^{-1} LPS for 60 min prior to a 15 min challenge with fMLP (100 nM). Superoxide anion release was measured with a spectrophotometric cytochrome C reduction assay and expressed as nmol superoxide anion generated/10^6 cells. Values represent mean ± s.e.mean from 10 experiments each carried out in triplicate. $^*P < 0.005$, significantly different from fMLP alone.

2.5 nM [^{3}H]-$InsP_6$ in either the above intracellular-like binding buffer or in 25 mM HEPES-buffered PBS containing 1 mM $CaCl_2$ and $MgCl_2$ (pH 7.5) produced a marked reduction in specific [^{3}H]-$InsP_6$ binding (76 ± 0.8% and 74 ± 1.2% respectively) compared to that observed in membranes. However, since assessment of cell viability demonstrated that approximately 10% of the pelleted neutrophils were trypan blue positive, an alternative separation method was followed using centrifugation through an inert oil cushion. Using this protocol, < 0.03% of the [^{3}H]-$InsP_6$ added was associated with the cell pellet irrespective of the incubation buffer used. Under identical conditions, and in the same experiment, > 16% specific [^{3}H]-LTB_4 binding was observed ($n = 2$, data not shown).

Table 1 Effects of TNFα and PAF on unstimulated and fMLP-induced superoxide anion generation in human neutrophils

	Superoxide anion generation (nmol/10^6 cells)	
	Unstimulated	*Stimulated*
Control	0.56 ± 0.08	3.50 ± 0.26
TNFα	0.88 ± 0.05	18.04 ± 2.33
PAF	0.78 ± 0.11	20.42 ± 2.16

Human neutrophils were suspended in PBS containing 25 mM HEPES as detailed in the Methods section, and preincubated with TNFα (200 u ml^{-1}, 30 min) or PAF (100 nM, 5 min) prior to a 15 min treatment with fMLP (100 nM). Superoxide anion release was assessed spectrophotometrically by a cytochrome C reduction assay and expressed as nmol superoxide anion generated/10^6 cells. Values represent mean ± s.e.mean of 3 separate experiments, each performed in triplicate.

Effect of $InsP_6$ on fMLP-stimulated shape change and superoxide anion generation

In view of the above data indicating the absence of true extracellular $InsP_6$ receptors in neutrophils, we sought to re-evaluate the functional effects of $InsP_6$ in these cells using respiratory burst activity and shape change as activation indices. The effect of $InsP_6$ on basal and fMLP-induced shape change was used as a sensitive indicator of potential chemotactic (Qu *et al.*, 1995) and priming (Haslett *et al.*, 1985) activity and also to determine the optimal $InsP_6$ preincubation period required for subsequent superoxide anion-priming experiments. Figure 4a illustrates the effects of incubating unprimed neutrophils with 100 μM $InsP_6$ for 0.5–30 min on basal and submaximal (0.1 nM) fMLP-induced neutrophil shape-change. $InsP_6$ (100 μM), unlike other established priming agents (Figure 5), had no effect on basal shape change (Figure 4a), but did cause a small and transient enhancement (26 ± 1.2% at 30 s) of fMLP-induced shape change (Figure 4a). TNFα, PAF and LPS did not enhance fMLP (100 nM)-induced shape change (data not shown). This pattern of effects (ie. transient enhancement of fMLP-induced shape change, but no effect of $InsP_6$ alone) correlates well with the time course effects of $InsP_6$ on fMLP-induced superoxide anion release reported by Eggleton & colleagues (1991) but is not observed with LPS, TNFα or PAF and hence appears to be unique to this priming agent (Young *et al.*, 1990). $InsP_6$ (100 μM, 30 s) also caused a small leftwards shift in the concentration-response curve for fMLP-induced shape change (fMLP alone, EC_{50} 76 pM; fMLP + $InsP_6$, EC_{50} 33 pM, $P < 0.01$).

The ability of $InsP_6$ to prime human neutrophils for enhanced fMLP-stimulated superoxide anion release was compared to the effects of lipopolysaccharide (100 ng ml^{-1}, 60 min), a well established neutrophil priming agent. $InsP_6$ alone (100 μM, 30 s) had no effect on basal superoxide anion release and caused only a very minor (1.8 ± 0.3 fold, $P < 0.005$, $n = 4$) enhancement of fMLP-stimulated superoxide anion generation compared with LPS (6.8 ± 0.6 fold, $P < 0.005$, $n = 4$) (Figure 6). This degree of priming of the fMLP-stimulated superoxide anion response by $InsP_6$ is very similar to that reported by Eggleton *et al.* (1991). In a separate series of experiments TNFα (200 u ml^{-1}, 30 min) and PAF (100 nM, 5 min) also enhanced fMLP-induced superoxide anion generation to a considerably greater extent than observed formerly with $InsP_6$ (Table 1).

Discussion

Neutrophils play a key role in defending the body against infection. However, the enormous histotoxic capacity of these cells dictates that uncontrolled or inappropriate activation can cause significant host tissue damage. One of the most important control steps involved in regulating respiratory burst activity is the requirement for the neutrophil to be primed before it will respond to a secretagogue challenge. While a wide variety of cell- and bacterial-derived products (eg. granulocyte-macrophage colony stimulating factor, PAF, TNFα and LPS) and physicochemical insults (eg. hypotonic challenge) can prime neutrophils, the specific intracellular mechanisms responsible for this process are yet to be fully defined.

Recently, $InsP_6$, a ubiquitous and abundant cytosolic inositol polyphosphate (Bunce *et al.*, 1993; Stuart *et al.*, 1994), was identified as a novel neutrophil priming agent, being able to facilitate fMLP-induced superoxide anion release without affecting basal superoxide anion generation (Eggleton *et al.*, 1991). In this study, preincubation of human neutrophils with $InsP_6$ (up to 250 μM) had no effect on basal superoxide anion generation but caused a 2 fold enhancement of the response to fMLP (2 μM). This led to the proposal that $InsP_6$, released from dying or effete cells at an inflammatory focus, may serve to augment local neutrophil respiratory burst activity. Our experiments sought to identify whether this effect of $InsP_6$ is receptor-mediated and re-evaluate its priming potential relative to other more established agents. Our data indicate that while specific, low affinity [^{3}H]-$InsP_6$ binding can be detected in neutrophil membranes, intact cells do not bind [^{3}H]-$InsP_6$, and that the absolute priming effect of $InsP_6$ is extremely weak and short-lived in comparison to other priming agents such as LPS and granulocyte macrophage colony stimulating factor, where the priming effect lasts for several hours (Balazovich *et al.*, 1991).

Analysis of [^{3}H]-$InsP_6$ binding to neutrophil membranes demonstrated the presence of at least two low affinity binding sites (K_D values of 0.15 and 5 μM), and displayed only a 3 fold selectivity for $InsP_6$ over Ins(1,3,4,5,6)P_5. These data contrast to the readily saturable, high affinity [^{3}H]-$InsP_6$ binding previously reported in, for example, rat cerebellum (Hawkins *et al.*, 1990), bovine adrenal chromaffin cells (Regunathan *et al.*, 1992) and canine cardiac microsomes (Kijima & Fleischer, 1992), and suggest that $InsP_6$ binding in human neutrophils may not reflect an interaction with any of the currently identified membrane-associated $InsP_6$ binding sites: these include the G-protein receptor regulatory protein arrestin (Regunathan *et al.*, 1992; Palczewski *et al.*, 1991), the IGF-II receptor (Kar *et al.*, 1994), the Golgi K^+ channel coatomer (Fleischer *et al.*, 1994) and the α-subunit of the clathrin assembly protein AP-2, recently identified as the $InsP_6$ receptor in rat cerebellum (Volgmaier *et al.*, 1992). This latter molecule is a 300–350 kDa protein involved in the formation of clathrin-coated vesicles at the plasma membrane, and is comprised of multiple subunits, including two doublets of 115 kDa and 105 kDa, which bind $InsP_6$ with a K_D of 12 nM (Theibert *et al.*, 1992), and two non-binding singlets of 50 and 17 kDa.

The pH-dependency of [^{3}H]-$InsP_6$ binding in neutrophil membranes also differs from that obtained in rat cerebellum (Theibert *et al.*, 1992) and rat cerebral cortex (Nicoletti *et al.*, 1990), where maximal binding occurred at pH 7 and 6, respectively. In addition, a pH optimum of 8, with marked inhibition of [^{3}H]-$InsP_6$ binding observed at more alkaline values, makes a simple charge-based membrane interaction unlikely. The ability of Mg^{2+} to potentiate [^{3}H]-$InsP_6$ binding in neutrophil membranes is qualitatively very similar to findings reported in rat cerebellum, where multivalent cations (Mg^{2+} and trace amounts of contaminating Fe^{3+} and Al^{3+}) augmented specific [^{3}H]-$InsP_6$ binding, possibly by acting as bridges between $InsP_6$ and negatively charged membrane phospholipid phosphates (Poyner *et al.*, 1993).

A variety of potential non-receptor mechanisms may underly the ability of $InsP_6$ to function as a weak priming agent. For example, it has recently been shown that negatively charged agents *per se* potentiate superoxide anion generation (Miyahara *et al.*, 1993) and also that $InsP_6$ can inhibit CD62-L (L-selectin)-mediated adherence of neutrophils to activated endothelial cells (Cecconi *et al.*, 1994). It is uncertain however how relevant this latter observation is to the priming effect of $InsP_6$ since cross-linking of CD62-L has recently been reported to induce rather than inhibit, neutrophil priming (Waddell *et al.*, 1994). It is also clearly possible that the powerful Ca^{2+} chelation properties or other, as yet unidentified, effects of $InsP_6$ may perturb neutrophil homeostasis. It should be noted however, that the studies of Eggleton and co-workers (1991) indicated that a similar priming effect is not observed with the lower inositol polyphosphates including Ins(1,3,4,5,6)P_5.

In summary, this study provides evidence for specific, low affinity, membrane associated [^{3}H]-$InsP_6$ binding in human neutrophils that is pH-dependent, heat-labile, augmented by Mg^{2+}, Ca^{2+} and Na^+ and located intracellularly. $InsP_6$, released from damaged or necrotic cells at an inflammatory focus, may interact with the neutrophil surface in a non-receptor-mediated fashion, to cause priming of NADPH oxidase function and polarization responses, but these effects are modest in comparison to other established priming agents.

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Demonstration of Reversible Priming of Human Neutrophils Using Platelet-Activating Factor

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Exposure of neutrophils to agents such as lipopolysaccharide, tumor necrosis factor-α (TNF-α), and the granulocyte-macrophage colony-stimulating factor causes a major upregulation of subsequent agonist-induced NADPH oxidase activation. This priming effect is a prerequisite for neutrophil-mediated tissue damage and has been widely considered to be an irreversible process. We have investigated the potential for neutrophils to recover from a priming stimulus by studying the effects of platelet-activating factor (PAF). PAF did not stimulate respiratory burst activity directly, but caused a rapid (maximal at 10 minutes) and concentration-dependent (EC_{50} 50.2 nmol/L) increase in N-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated superoxide anion release. At time-points >10 minutes, this priming effect spontaneously declined, with return to basal levels of fMLP-stimulated superoxide anion generation by 120 minutes. An identical priming time-course was observed with N-methyl carbamyl PAF, a non-metabolizable analogue of PAF, indicating that the transient nature of PAF-induced priming was not secondary to PAF metabolism. Two structurally diverse PAF receptor antagonists (UK-74,505 and WEB 2086), added 10 minutes after PAF addition, increased the rate of decay of the priming effect. In contrast, TNF-α–induced priming, which was of a similar magnitude to that observed for PAF, was slower to evolve (maximal at 30 minutes) and remained constant for at least 120 minutes. The reversible nature of PAF-induced priming was confirmed by demonstrating that PAF-, but not TNF-α–, induced cell polarization (shape change) and CD11b-dependent neutrophil binding of albumin-coated latex beads was also transient, with return to basal, unstimulated levels by 120 minutes. Furthermore, cells that had spontaneously deprimed following PAF exposure retained their capacity to be fully reprimed by a subsequent addition of either PAF or TNF-α. These data imply that neutrophil priming is not an irreversible event: the demonstration of a cycle of complete priming, deprimming, and repriming offers the potential for functional recycling of neutrophils at sites of inflammation.

NEUTROPHILS PLAY a fundamental role in the acute inflammatory response, destroying invading microbial pathogens and thereby minimizing infection of the host. The response of neutrophils to various proinflammatory stimuli is largely determined by their previous exposure to agents such as cytokines (eg, tumor necrosis factor-α [TNF-α], and interleukin-8, [IL-8]), lipid mediators (eg, platelet-activating factor [PAF] and leukotriene B_4 [LTB_4]) or bacterial products (eg, N-formyl-methionyl-leucyl-phenylalanine [fMLP], and lipopolysaccharide [LPS]). At physiologically relevant concentrations these agonists elicit cell polarization, recruitment, and activation of cell-surface β_2-integrins (eg, CD11b/CD18), and enhance, or "prime," neutrophil responses (eg, phagocytosis, respiratory burst activation, degranulation) to other secretagogue agonists.[1-4] In vivo data suggest that priming also plays a critical role in the recruitment of neutrophils to an inflammed site.[5] However, although this switching of neutrophils from a relatively unresponsive to a hyperresponsive state is a prerequisite for physiological neutrophil-mediated bacterial destruction, it may, if uncontrolled, lead to neutrophil-mediated tissue damage.[6]

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Previous studies have demonstrated that human peripheral blood neutrophils, incubated with the priming agents granulocyte colony-stimulating factor G-CSF or LPS, maintain an enhanced superoxide anion secretory response to fMLP for at least 24 hours.[7] Studies undertaken in vivo have demonstrated that sheep peripheral blood and bone marrow-derived neutrophils also remain primed for at least 24 hours following endotoxin LPS infusion, suggesting that maintenance of the primed state is an important part of the long-term inflammatory response to endotoxin.[8] These observations have been widely interpreted as indicating that neutrophil priming is an essentially irreversible phenomenon.[8]

Despite its obvious importance to the regulation of inflammation, little attention has been given to the potential for neutrophils, once primed, to revert to their former quiescent state, ie, to be "deprimed." However, in two studies where neutrophil priming has been induced using physicochemical stimuli ie, hypotonic treatment,[9] or exposure to the highly-charged Ca^{2+}-chelator, inositol hexakisphosphate,[10] priming of the superoxide anion response appeared to be transient. The lack of study in this area may, at least in part, reflect the difficulties encountered in isolating and maintaining these cells in an unprimed state ex-vivo, or ensuring complete removal or antagonism of the initial priming signal. In addition, isolated neutrophils have a relatively short lifespan due to their high rate of constitutive programmed cell death or apoptosis.[11,12] For these reasons, most in vitro studies have focused on the short-term effects of priming agents and the cellular mechanisms responsible for such events.

In view of the potential pathophysiological, and hence therapeutic, importance of being able to rescue neutrophils from the primed state, we have investigated the possible reversibility of neutrophil priming induced by physiological, receptor-mediated priming agents. Using human peripheral blood neutrophils incubated at a concentration of PAF that causes a rapid enhancement of fMLP-stimulated respiratory burst activity without any direct effect on superoxide anion release, we observed a spontaneous and complete decay of

the PAF-primed superoxide anion response and PAF-induced CD11b/CD18 activation and to a lesser extent, PAF-induced shape change. The rate of neutrophil recovery following PAF addition could be enhanced by the use of selective PAF receptor antagonists (WEB 2086 and UK-74,505). Furthermore, the deprimed cells retained their full capacity to be reprimed by an alternative priming agent (TNF-α) or a further addition of PAF. The ability of neutrophils to participate in a complete priming/depriming/reprimming cycle allows far greater flexibility in the control of neutrophil behavior at an inflammatory site than was hitherto realized.

MATERIALS AND METHODS

Neutrophil preparation. Peripheral venous blood was taken from healthy adult volunteers, anticoagulated with 4 mL 3.8% sodium citrate/40 mL blood, and centrifuged (300*g*) for 20 minutes. Neutrophils were isolated exactly as previously detailed[2] using dextran sedimentation and discontinuous plasma-Percoll gradients. This isolation technique yields neutrophils that display very low levels of basal shape change (<8% assessed flow-cytometrically) or direct fMLP-induced superoxide anion generation. The purified neutrophils were washed sequentially in platelet-poor plasma, phosphate-buffered saline (PBS) without, and PBS with, $CaCl_2$ and $MgCl_2$. Cell purity and viability (trypan blue exclusion) were routinely >95% (<0.5% monocyte contamination) and >99.5%, respectively.

Shape change assay. Neutrophils (10^6 in 90 μL PBS containing $CaCl_2$ and $MgCl_2$) were equilibrated in a gently shaking water-bath for 5 minutes at 37°C. Priming agents were added in a 10-μL volume to achieve the required drug concentrations (PAF 1 nmol/L to 10 μmol/L, TNF-α 200 U/mL) and incubations continued for the periods stated. Preliminary experiments demonstrated this concentration of TNF-α to be optimal in causing maximal enhancement of fMLP-induced superoxide anion generation with minimal direct respiratory burst activation (data not shown). To determine the IC_{50} values of the PAF receptor antagonists used, neutrophils were incubated with WEB 2086 or UK-74,505 (both at 10 nmol/L to 10 μmol/L) for 30 minutes before the addition of priming agents. For investigations examining the reversibility of PAF-induced priming, neutrophils were treated with PAF (1 μmol/L) for 10 minutes before addition of 1 μmol/L WEB 2086 or UK-74,505. fMLP (100 nmol/L) or buffer (PBS) was added to samples (final volume 1 mL) 10 minutes before the addition of an equal volume of 2.5% glutaraldehyde. Samples were analyzed for shape change by flow cytometry (Coulter EPICS Profile II; Coulter Electronics, Luton, UK) using a slight modification of a previously published method.[13] Percentage shape change was calculated from the mean forward light scatter of each sample by gating on the non-shape changed neutrophil population. The values obtained using this method correlate closely with those derived by direct visual assessment of shape change, with the exception that the flow cytometric method of assessment slightly overestimates the extent of basal shape change.[13]

Superoxide anion release assay. Neutrophils were isolated, equilibrated at 37°C, and incubated with PAF or PBS exactly as detailed above, except that cytochrome C (800 μL, 1 mg/mL) was added immediately before the addition of fMLP. One of each set of quadruplicate determinations included superoxide dismutase (375 U) to allow confirmation of the specificity of cytochrome C reduction. Reactions were stopped by placing the cells on ice, followed by centrifugation (12,500*g*, 2 minutes, 4°C). The superoxide dismutase-inhibitable reduction of cytochrome C was determined in each supernatant by measuring the peak absorbance between 535 and 565 nm using a Pye-Unicam scanning spectrophotometer, and expressed as nanomoles superoxide anions generated per 10^6 neutrophils. In experiments designed to assess the ability of PAF-recovered neutrophils to be reprimed with either TNF-α or a further addtion of PAF, cells were treated with PAF (1 μmol/L) or PBS for 120 minutes, before a final incubation with PAF (1 μmol/L, 10 minutes) or TNF-α (200 U/mL, 30 minutes) and assessment of fMLP-stimulated superoxide anion release.

To examine the effects of hypotonic challenge on neutrophil function, cells (10^6 in 250 μL PBS) were equilibrated at 37°C as outlined above and incubated for 19 minutes in PBS containing 80 μmol/L cytochrome C, with 150 mmol/L NaCl (isotonic incubations) or 50 mmol/L NaCl (hypotonic incubations). Neutrophils were then treated for 1 minute with 20 μL of either 5 mol/L NaCl (to reverse hypotonicity to isotonicity) or PBS (to retain hypotonicity or isotonicity). The effects of the hypotonic challenge itself on basal and fMLP-stimulated superoxide anion generation, together with the ability of these cells to deprime following restoration of isotonicity and thereafter be reprimed with PAF, was then assessed as detailed above.

Neurophil adhesion to albumin-coated latex beads (ACLB). Fluorescent latex beads (2.5% packed vol/vol stock solution as purchased) were washed (three times) in PBS, resuspended at 2.5% (vol/vol) in PBS containing 10 mg/mL human serum albumin, and incubated for 10 minutes at 25°C. The resultant ACLB were again washed (three times) in PBS and finally resuspended at 0.75% (vol/vol). Neutrophils (175 μL aliquots at 10^7/mL, in PBS with $CaCl_2$ and $MgCl_2$) were incubated in a shaking water-bath at 37°C for 5 minutes, and then treated with PAF (1 μmol/L), TNF-α (200 U/mL) or PBS (all added in a 15-μL volume) for 0 to 120 minutes. ACLB (25 μL of 0.75% vol/vol solution) were added to each tube 15 minutes before the termination of each reaction, except for time points <15 minutes where the beads were added before the agonist. Neutrophils were then fixed by the addition of 0.5 mL of 0.5% glutaraldehyde. After 30 minutes at room temperature, nonadherent ACLB were removed by washing with PBS (three times) and bead-binding to the neutrophil assessed using an EPICS Profile II (Coulter Electronics), as previously detailed.[14]

Statistics. All values are expressed as means ± standard error of mean (SEM) of (n) number of separate experiments. Values, where applicable, were compared by ANOVA or the Student's *t*-test for paired data, with $P < .05$ considered to be significant. Significant differences between groups were determined by the Newman-Keuls procedure.

Materials. fMLP, PAF, superoxide dismutase (SOD), cytochrome C, PBS (with or without $CaCl_2$ and $MgCl_2$), dextran-500, Percoll, human serum albumin and glutaraldehyde (25%) were purchased from Sigma (Poole, UK). TNF-α was obtained from Genzyme (Cambridge, MA). One micron fluorescent microspheres were purchased as a 2.5% solids-latex (2.5% vol/vol) stock solution from Polysciences Inc through the UK supplier Park Scientific (Nottingham, UK). WEB 2086 and UK-74,505 were gifts from Boehringer Ingelheim Ltd (Berks, UK) and Dr J. Parry (Pfizer, Sandwich, UK), respectively. 1-*O*-alkyl-2-*N*-methylcarbamyl-glycerophosphocholine (N-methyl carbamyl PAF) was obtained from Calbiochem (Nottingham, UK).

RESULTS

PAF is an established and important inflammatory mediator in vivo.[15] It was selected for this study because its priming effect in neutrophils is rapid, receptor-mediated,[16] associated with minimal direct activation of superoxide anion release,[17,18] and inhibitable by specific, high-affinity PAF receptor antagonists. Under our own experimental conditions, PAF (1 nmol/L to 10 μmol/L) did not affect spontaneous superoxide anion release (Fig 1A), but caused a rapid (maximal at 10 minutes), concentration-dependent increase in fMLP-stimulated superoxide anion release (EC_{50} 50.2 nmol/

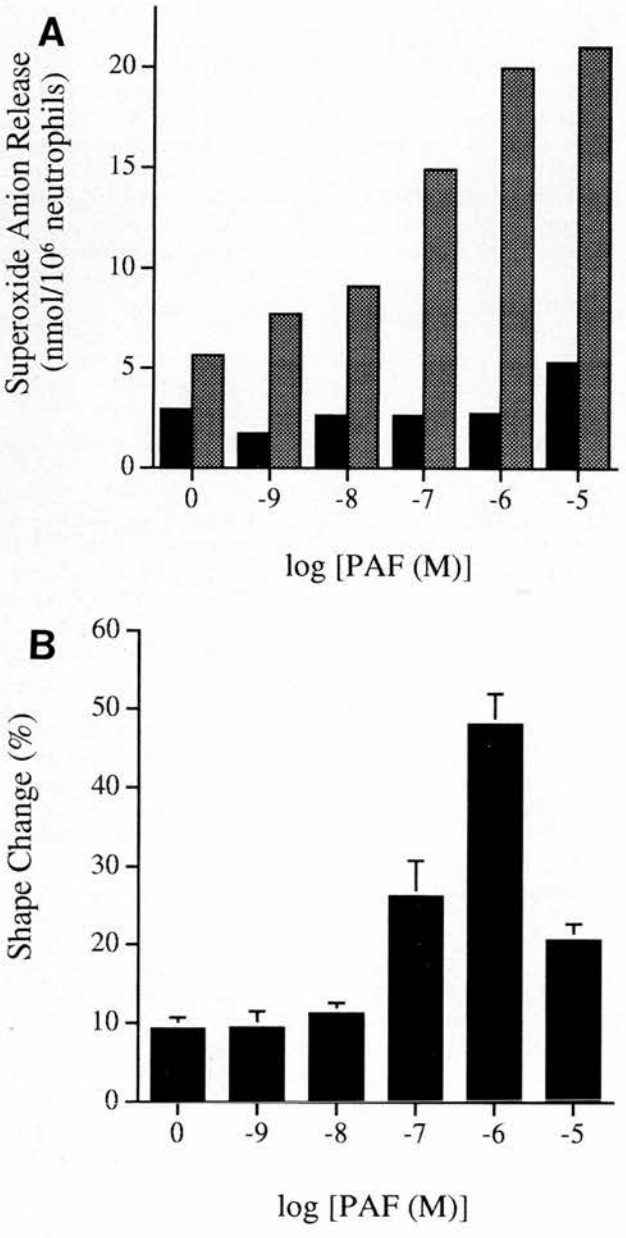

Fig 1. Effect of PAF on basal and fMLP-stimulated superoxide anion generation and shape change in human neutrophils. (A) Concentration-response data for superoxide anion generation. Isolated human neutrophils (10^6 in 90 μL PBS) were equilibrated for 5 minutes at 37°C and incubated with 10 μL of PAF (10 nmol/L to 10 μmol/L final concentration) for 10 minutes. Cells were then stimulated with 100 nmol/L fMLP (hatched bars) or buffer (closed bars) for 10 minutes in the presence of cytochrome C (1 mg/mL), in a final volume of 1 mL. Reactions were terminated by placing samples on ice and superoxide anion release was assessed by scanning spectrophotometry. Values represent mean of triplicate determinations from a single experiment, representative of six. (B) Concentration-response data for shape change. Neutrophils were incubated as outlined above for superoxide anion generation, except that buffer replaced the cytochrome C and reactions were terminated by the addition of 1 mL 2.5% glutaraldehyde. Samples were analyzed by flow cytometry and percent shape change calculated from the mean forward light scatter values, by gating on the non-shape changed population. Values represent mean ± SEM for six independent experiments each performed in duplicate.

L, Fig 1A), and a similar concentration-dependent increase in shape change (EC_{50} 110 nmol/L, Fig 1B). In the shape change experiments, 10 μmol/L PAF appeared to have less effect than 1 μmol/L PAF, but this correlated with the light-microscopic observation of large, round, "glassy"-looking cells, suggestive of cell swelling. In view of these findings, a PAF concentration of 1 μmol/L was chosen for all further priming studies.

PAF-induced priming of superoxide anion generation in neutrophils is reversible. Figure 2 illustrates how the length of the initial PAF incubation period affects the subsequent enhancement of superoxide anion release in response to fMLP. The ability of PAF to prime the fMLP-response was maximal after a 10-minute PAF preincubation, with the priming effect decaying thereafter, to approach unprimed levels by 2 hours (Fig 2). Notably, the pattern of reversal of the PAF priming effect was consistently biphasic, with an initial rapid loss in priming occurring within 15 to 30 minutes of PAF addition, followed by a second, slower phase of decay. This progressive decline in the magnitude of the primed fMLP-superoxide anion response was not due to cell necrosis, as viability (assessed by trypan blue exclusion) was routinely >95% for all time points studied.

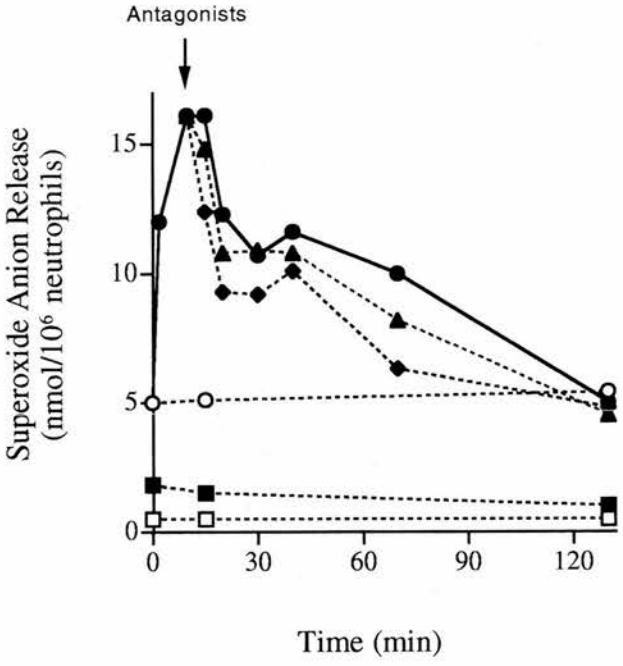

Fig 2. Time-course for PAF-priming of fMLP-stimulated superoxide anion release in human neutrophils. Isolated human neutrophils (10^6 in 80 μL PBS) were equilibrated for 5 minutes at 37°C and incubated with 10 μL PAF (1 μmol/L) (closed symbols) or buffer (open symbols) for 10 minutes. A 10-μL aliquot of WEB 2086 (1 μmol/L, triangles), UK-74,505 (1 μmol/L, diamonds), or buffer (circles) was added 10 minutes after PAF. Cells were incubated for a further 0 to 120 minutes before a final 10-minute stimulation with 100 nmol/L fMLP (circles, triangles, diamonds) or buffer (squares) in the presence of cytochrome C (1 mg/mL). Reactions were terminated at the appropriate times by placing the cells on ice and superoxide anion release assessed by scanning spectrophotometry. Data points represent mean values for triplicate determinations from three separate experiments. SEM values were all <10 % of mean and are omitted for reasons of clarity.

In an attempt to elucidate some of the possible factors responsible for this time-dependent reversal of PAF-mediated neutrophil priming, additional experiments were undertaken to assess the role of PAF metabolism and PAF receptor desensitization. Firstly, PAF was substituted by a nonmetabolizable analogue, N-methyl carbamyl PAF. This resulted in a near identical time course (data not shown) to that illustrated in Fig 2, indicating that PAF degradation was not responsible for the loss in the priming effect. Secondly, we examined the ability of two specific, but structurally different, PAF receptor antagonists, WEB 2086 and UK-74,505, to influence the rate of depriming. Preliminary studies established optimal conditions for the use of these antagonists: a 30-minute preincubation with UK-74,505 caused a concentration-dependent (IC_{50} 68 nmol/L) and complete (at 1 μmol/L) inhibition of the PAF-primed superoxide anion response, whereas inhibition by WEB 2086 was biphasic and incomplete (55% $\pm$ 4% inhibition with 10 μmol/L WEB 2086, data not shown). Neither of these compounds, at the concentrations used, affected neutrophil viability nor superoxide anion release in control or fMLP-treated cells (data not shown). Thus, to investigate the influence of PAF receptor blockade on the rate of decay of PAF-induced priming, both antagonists were used at a concentration of 1 μmol/L. When WEB 2086 (1 μmol/L) or UK-74,505 (1 μmol/L) was added 10 minutes after PAF, a small but significantly faster ($P < .05$) rate of decay of the PAF-primed superoxide anion response was observed (Fig 2), with UK-74,505 having the greater effect. This data suggests that although PAF receptor desensitization or uncoupling may play a role in the decay of the priming effect, this process is not complete following a 10-minute incubation with 1 μmol/L PAF.

In marked contrast to the priming time course observed with PAF, TNF-α–induced priming, although slower to evolve (maximal at 30 minutes), remained constant for at least 2 hours (nanomoles superoxide anion released: at 30 minutes: fMLP (100 nmol/L) 4.6 $\pm$ 1.6, TNF-α (200 U/mL) + fMLP 20.1 $\pm$ 3.2; at 2 hours: fMLP 5.0 $\pm$ 0.7, TNF-α + fMLP 19.2 $\pm$ 4.9, n = 3).

Transient effects of PAF on neutrophil shape change and ACLB binding. To validate our observations of neutrophil recovery following PAF-mediated priming of the respiratory burst, we undertook further time course studies to examine the effects of PAF on neutrophil shape change and CD11b activation. Shape change was chosen because previous studies have demonstrated a tight correlation between priming of the superoxide anion response and the extent or proportion of neutrophils that have undergone cell polarization.[2] In addition, the relatively weak priming effect of IL-8 on superoxide anion generation has been reported to partially reverse with time without any concomitant recovery of CD11b expression.[19]

Incubation of neutrophils with 1 μmol/L PAF caused a rapid (maximal at 2 minutes) increase in shape change, which then declined spontaneously towards basal levels by 30 minutes (Fig 3). The subsequent small increase in percent shape change, observed between 30 to 120 minutes, paralleled the changes seen in control cells. The extent of the initial shape change response to PAF was similar to that observed following a 10-minute incubation with 100 nmol/L fMLP (80.8% $\pm$ 7.1%, n = 3). It should be noted here that flow-cytometric quantification

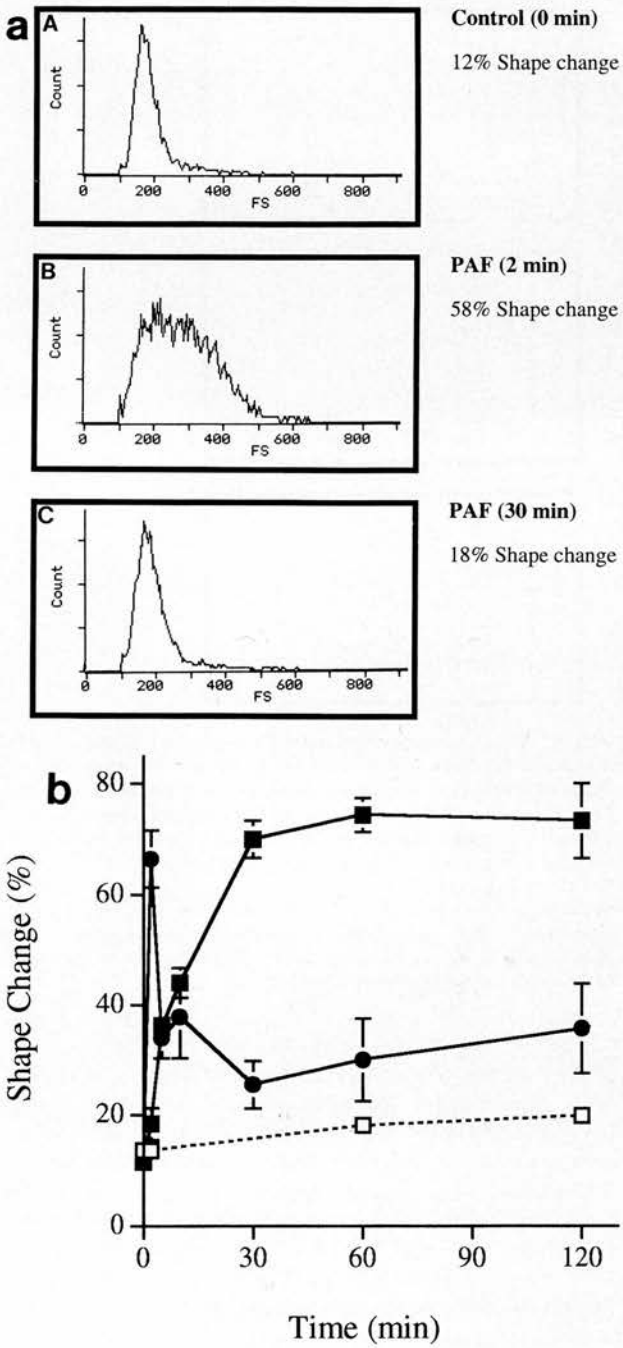

Fig 3. Time-course for PAF- and TNF-α–induced shape change in human neutrophils. (a) Representative flow-cytometry (EPICS Profile II) histograms of control cells (A) and cells incubated with PAF for 2 minutes (B) or 30 minutes (C) (x-axis shows mean forward light scatter (FS) and y-axis shows relative cell number). Percent shape change was calculated from the FS of each sample by gating out the population of non-shape changed neutrophils determined from control samples. (b) Time-course data for PAF- and TNF-α–induced shape change. Isolated human neutrophils (10^6 in 90 μL PBS) were equilibrated for 5 minutes at 37°C and incubated with 10 μL PAF (1 μmol/L) (closed circles), TNF-α (200 U/mL) (closed squares), or buffer (open squares) for 0 to 120 minutes. Reactions were terminated at the appropriate times by addition of an equal volume of 2.5% glutaraldehyde. Samples were analyzed by flow cytometry and percent shape change calculated as detailed above. Values represent mean $\pm$ SEM for four independent sets of duplicate determinations. Where not shown, SEM values fall within the symbols.

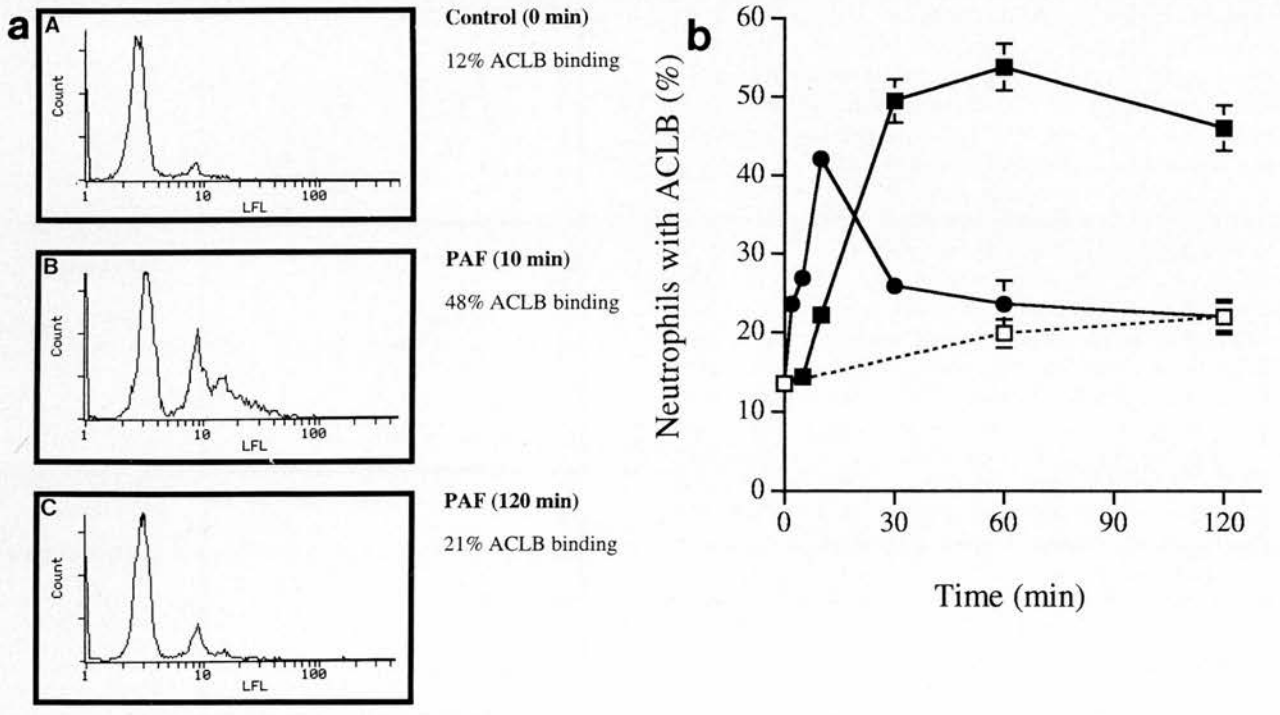

Fig 4. Time-course for PAF- and TNF-α–induced binding of ACLB. (a) Representative flow-cytometry histograms of control cells (A) and cells incubated with PAF for 10 minutes (B) or 120 minutes (C) (x-axis shows logarithmic scale green fluorescence (LFL) and y-axis shows relative cell number). The lowest fluorescent (far left) peak represents neutrophils with no attached ACLB then, with increasing binding, single, double, and triple bead peaks (correlated by fluorescence microscopy) can be distinguished. The percent neutrophils with attached ACLB was calculated by gating out the far left peak determined from time-matched control samples. (b) Time-course data for PAF- and TNF-α–induced binding of ACLB. Isolated human neutrophils (1.75×10^6 in 175 μL PBS) were equilibrated for 5 minutes at 37°C and incubated with 15 μL PAF (1 μmol/L) (closed circles), TNF-α (200 U/mL) (closed squares), or buffer (open squares) for 0 to 120 minutes. ACLB (25 μL) were added 15 minutes before termination of the reaction with 0.5 mL 0.5% glutaraldehyde, except for time points <15 minutes where the beads were added before the agonist. Samples were analyzed by flow cytometry and the percent neutrophils with attached ACLB was calculated, as detailed above. Values represent mean ± SEM for four independent sets of determinations, each performed in duplicate. Where not shown, SEM values fall within the symbols.

of shape change, although a convenient and highly reproducible assay, gives consistently higher levels of basal shape change (approximately 8%[13]) than those obtained by direct visual assessment of cell morphology. Our data confirm, however, that PAF-induced shape change, like priming of the superoxide anion response, is a transient event. Neutrophil shape change to TNF-α (200 U/mL) was of a similar magnitude (70.1% ± 1.8%, n = 4) to that induced by PAF, but was slower to evolve (plateau at 30 minutes) and remained constant for the 2-hour incubation period (Fig 3).

As a third index of priming, we followed the β_2-integrin–dependent binding of ACLB over a 2-hour incubation with either PAF or TNF-α. Again, PAF (1 μmol/L) induced a time-dependent increase in ACLB binding, maximal after 10 minutes, which declined to reach control levels by 2 hours (Fig 4). TNF-α (200 U/mL) also augmented ACLB binding but, unlike PAF, the extent of bead binding reached a plateau at 30 minutes and remained constant for the ensuing 90-minute incubation period (Fig 4).

Repriming of neutrophils with PAF or TNF-α. We next investigated whether neutrophils that had primed and then spontaneously deprimed during a 120-minute incubation with PAF were capable of being reprimed. Figure 5 demonstrates that PAF-recovered neutrophils retain their full capacity to be reprimed when challenged again with either PAF (1 μmol/L, 10 minutes) or TNF-α (200 U/mL, 30 minutes), generating similar amounts of superoxide anions upon fMLP stimulation as freshly-primed cells. This finding was repeated when neutrophils were primed and deprimed using a modification of a previously described hypotonic challenge protocol.[9] Under isotonic conditions, fMLP (100 nmol/L) and PAF (1 μmol/L) alone elicited little superoxide anion release, with PAF enhancing the fMLP-stimulated superoxide anion response by 3.8-fold (Table 1). A 20-minute hypotonic challenge resulted in a modest (twofold) priming of the fMLP response, which recovered towards control levels when isotonicity was restored for 1 minute. Subsequent treatment with PAF reprimed the fMLP response, albeit to a slightly lower level than that observed in cells maintained under isotonic conditions. Cell viability was routinely >95% for all conditions studied. Thus, like cells that had deprimed following PAF exposure, osmotically primed and deprimed neutrophils also retained their capacity to be primed for a second time by a physiological agonist such as PAF.

DISCUSSION

The neutrophil can exist in a number of different functional states and this has a significant bearing on its behavior

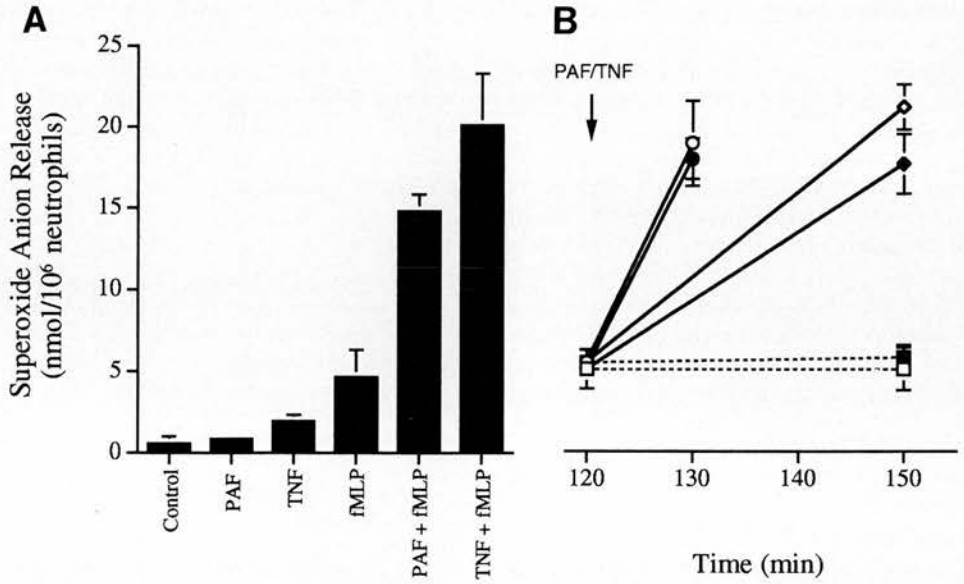

Fig 5. Repriming of human neutrophils with PAF and TNF-α following initial priming with PAF. (A) Superoxide anion priming with PAF and TNF-α in freshly isolated cells. Neutrophils (10^6 in 90 μL PBS) were equilibrated for 5 minutes at 37°C, and then incubated with 10 μL of buffer, PAF (1 μmol/L, 10 minutes) or TNF-α (200 U/mL, 30 minutes), as these represent optimal priming conditions for later comparisons with reprimed neutrophils (see [B]). Cells were subsequently stimulated with 100 nmol/L fMLP or buffer for 10 minutes, in the presence of cytochrome C (1 mg/mL), in a final volume of 1 mL. Superoxide anion release was finally assessed spectrophotometrically. (B) Superoxide anion repriming of neutrophils with PAF and TNF-α following a 120-minute incubation with PAF. Neutrophils (10^6 in 80 μL PBS) were incubated with 1 μmol/L PAF (closed symbols) or buffer (open symbols) for 120 minutes, followed by a second optimal priming challenge with PAF (1 μmol/L, 10 minutes, circles), TNF-α (200 U/mL, 30 minutes, diamonds), or buffer (30 minutes, squares). All samples were then stimulated with fMLP (100 nmol/L, 10 minutes) in the presence of cytochrome C (1 mg/mL), in a final volume of 1 mL, and analyzed for superoxide anion release, as above. Values represent mean ± SEM for triplicate determinations from three independent experiments.

and responsiveness in vitro. Thus, in the unprimed state, the neutrophil displays little or no secretory response when incubated with an agent such as fMLP, whereas such a challenge in a fully primed cell results in an explosive increase in respiratory burst activity; this priming-activation axis has been shown to be a major determinant of neutrophil behavior in vivo.[20] However, the very protracted priming effect of agents such as LPS, G-CSF, and GM-CSF, together with the short life-span of the neutrophil, has led to the belief that priming is a largely irreversible process.[7,8] Indeed, the sustained nature of the priming effect has been postulated to play a fundamental role in the long-term inflammatory response observed with certain agents, including endotoxin.[8] In this report, we provide evidence that neutrophil priming is not an irreversible process and, moreover, that these cells, once deprimed, can go through a further complete cycle of priming and activation.

Table 1. Effect of Transient Hypotonic Challenge on Basal, fMLP-Stimulated and PAF-Primed Superoxide Anion Generation in Human Neutrophils

	Isotonic	Hypotonic	Hypo-Isotonic
Control	2.8 ± 0.5	6.0 ± 0.4*	4.1 ± 0.6
PAF (1 μmol/L)	3.6 ± 0.4	7.1 ± 0.1*	5.7 ± 0.3
fMLP (100 nmol/L)	4.9 ± 0.6	10.1 ± 0.1*	6.0 ± 0.7
PAF + fMLP	18.9 ± 1.4	18.4 ± 0.5	11.5 ± 1.0*

Isolated peripheral blood neutrophils (10^6 in 250 μL PBS) were equilibrated for 5 minutes at 37°C and incubated for 19 minutes in PBS containing 150 mmol/L NaCl (isotonic incubations) or 50 mmol/L NaCl (hypotonic incubations). Neutrophils were then treated for 1 minute with 20 μL of either 5 mol/L NaCl (to reverse hypotonicity to isotonicity) or PBS (to retain hypotonicity or isotonicity). Superoxide anion release was assessed spectrophotometrically following incubation with buffer alone for 20 minutes (Control), buffer for 10 minutes followed by PAF for 10 minutes (PAF, 1 μmol/L), buffer for 10 minutes followed by fMLP for 10 minutes (fMLP, 100 nmol/L), or PAF for 10 minutes followed by fMLP for 10 minutes (PAF + fMLP). Values represent mean ± SEM for three separate experiments each performed in triplicate.

* $P < .05$ compared with values obtained under isotonic conditions.

The depriming of neutrophils observed following PAF treatment was apparent for fMLP-stimulated superoxide anion generation, CD11b function, and cell polarization, and hence was unlikely to represent selective downregulation of one particular component of the priming response, as reported with IL-8.[19] The ability of neutrophils to be reprimed by TNF-α and PAF after a 2-hour incubation, with maintenance of full viability throughout, excludes the possibility that the loss of the PAF priming effect was merely a consequence of the extended incubation procedure affecting cell integrity or metabolic status. While the basis for the decline in PAF-mediated priming is uncertain, the identical nature of the time-course of priming of superoxide anion release with N-methyl carbamyl PAF, a biologically active PAF analog that is completely resistant to metabolic inactivation by neutrophils or human serum,[21,22] makes PAF metabolism unlikely. We have also shown that inclusion of adenosine deaminase in these incubations does not influence the time-

course of PAF-primed superoxide anion responses, which excludes a secondary effect mediated via adenosine release and autocrine activation of cyclic adenosine monophosphate (AMP)-dependent protein kinase pathways (Kitchen, Rossi and Chilvers, unpublished observations, February 1995). Although homologous receptor desensitization may underlie the transient nature of the PAF signal, the increased rate of decay of fMLP-induced superoxide anion release following UK-74,505 or WEB 2086 addition and the return of a fully competent PAF priming response after 2 hours suggests that PAF receptor uncoupling/desensitization is both incomplete and transient. These data are consistent with previous studies demonstrating rapid activation-induced uncoupling and internalization of PAF receptors followed by subsequent receptor re-expression.[23] The recovery of PAF receptor number and function is likely to reflect extensive membrane attachment and metabolism of PAF (approximately 1 pmol/10^7 neutrophils/min).[24,25]

From our own comparisons of PAF and TNF-α and other published observations, it would appear that neutrophil priming in vitro falls into three categories: (1) fully reversible (eg, that induced by PAF, osmotic swelling, or inositol hexakisphosphate); (2) partially reversible (eg, with IL-1[7] or IL-8); or (3) largely irreversible (eg, with GM-CSF[19], G-CSF, or LPS). Further studies would be required to categorize TNF-α because its effects did not show any signs of recovery over the 2-hour incubation period used in this study, but have been reported to decay over 24 hours.[7] It is also intriguing to note that only agents in group (3) are able to modulate the rate of neutrophil apoptosis, which again testifies to the long duration of action of this class of agents.[26] While it is clear that neither the efficacy nor extent of the initial priming signal dictates the reversibility of the primed state (because PAF, TNF-α [Fig 5], LPS, and GM-CSF [data not shown] induce equivalent levels of priming), it is possible that the duration of the priming signal and/or its rate of onset are key determinants. However, in the absence of any clear mechanistic basis for neutrophil priming, it is also possible that the above agents use discrete signalling pathways to induce their priming effects.

This current observation of reversible priming may allow the pro-inflammatory, and potentially tissue-damaging, effects of neutrophil priming/activation to be counteracted by a process other than apoptosis or the pharmacological inhibition of neutrophil activation. It is unlikely that neutrophils within an inflammatory focus would be exposed in isolation to PAF or other "transient" priming agents. However, as endothelial cell-associated PAF has been shown to play a central role in neutrophil priming and migration through IL-1β–treated human umbilical vein endothelial cell monolayers in vitro,[27] and endogenously formed PAF is involved in leukocyte extravasation induced by IL-1 in vivo,[28] any delay in cell exit through an activated endothelial surface may permit cell recovery and the return of unprimed neutrophils to the circulation. Thus, the recognition that neutrophils have the potential to deprime allows an additional point of control in the early stages of the acute inflammatory response, whereby cells may return to their former quiescent state and potentially re-enter the circulating neutrophil pool. These deprimed neutrophils, once fully recovered, could again attain their maximal priming potential and mount subsequent responses, as dictated by ensuing inflammatory challenges. In contrast, priming agents with a longer duration of action would maintain neutrophils in the primed state for a much longer period of time and may, therefore, play a distinct role in vivo, in the wake of a more widespread or prolonged inflammatory insult.

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